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BACTERIAL OXIDATION OF SOME PRIMARY AMINES

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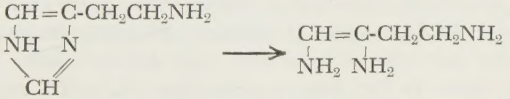
Primary amines produced from amino acids during putrefaction have been considered to be so resistant against bacteria that they remain unchanged in media for a long time. Lately, Miyaki and Hayashi (*1*) found, however, that some amines, such as histamine, were frequently destroyed in putrefying food and the amines that accumulated in it at the early stage disappeared rapidly on further progress of putrefaction, though each one decreased not always at the same rate and time.

Although the ability of mammalian organs to oxidize various amines is well known, there is relatively little information concerning the bacterial destruction of amines. More than thirty years ago, Koessler (*2*), and later Eggerth (*3*) too, observed that several types of bacteria were able to convert histamine to a substance giving a negative diazo-reaction and considered the change, probably analogous to the Bamberger reaction (*4*), to be in a following manner, without further evidences.



Beside the incorrectness of the postulated product, which can exist in a stable form only as its acyl derivatives, the imidazole ring might be split after the 4-aminoethyl group took change. Lately, Zeller (*5*) and Werle (*6*) stated that *Mycobacter, Pseudomonas* and *B. coli* seem to have an oxidase which acts on histamine and diamines widely, just as mammalian diamine oxidase (*26*), while Gale (*7*) reported that a strain of *B. coli* was able to oxidize putrescine but not histamine, showing an important disagreement. According to the latter worker, some amines, including mono- and di-amines, were oxidized almost completely by the adaptive enzyme systems, though the interrelations between each amine oxidizing system and their metabolic path were obscure. These have prompted us to study the bacterial oxidation of some re-

presentative primary amines, histamine, putrescine and isoamylamine, in more details to elucidate their metabolic paths and whether their deamination is catalysed by a system like mammalian amine oxidase or not.

EXPERIMENTAL

Materials—Imidazole-4-aldehyde and -carboxylic acid were prepared from hydroxymethyl-imidazole (*8*); imidazole-acetic acid from cyanomethyl-imidazole (*9*); imidazole-4-propionic acid from urocanic acid which was kindly offered from Prof. S. Akabori (*8*). Putrescine from adipic diamide and cadaverine from glutaraldehyde (*10*), were purified through their benzoyl derivatives. Isoamylamine and tyramine were prepared from leucine (purified through the copper salt) and tyrosine, respectively. γ-Aminobutyric acid was kindly offered by Mr. S. Sakurai. Synthetic isovaleric acid, given by Mr. S. Seno (*11*), was free from other organic acids unlike the usual commercial ones. Antihistamine and antiadrenaline drugs were kindly offered by Dr. S. Yamaoka. Other were purchased.

Bacteria—Bacteria were separated from soil by the enrichment technique using histamine as an only source of assimilable carbon and nitrogen as follows: An inoculum of soil suspensions in water was cultured at 27° until turbidity appeared. The culture medium contained 0.2 per cent histamine dihydrochloride, 0.2 per cent, KH_2PO_4 0.5 per cent NaCl, 0.01 per cent $MgSO_4 \cdot 7H_2O$, and was adjusted to pH 7.0. An inoculum of the growing culture was rapidly transfered to a new medium of the same composition and cultured at 27°. These procedures were repeated more than three times with an interval of 18 hours at the same temperature, during which the most active bacteria for histamine were selected. Finally they were spread on a plate and purified. It was kindly identified as *Achromobacter sp.* by Prof. K. Aiso.

Preparation of Cells for Experiments—An eighteen-hours broth culture at 27° was centrifuged and the cells were washed twice with cold distilled water. The suspensions were diluted with water to the same concentration (0.10±0.01 mg. cell-N per ml.) and taken for manometric experiments as the cells unadapted to a given amine. In some experiments, ammonium-glucose synthetic medium containing glucose 0.2 per cent, $(NH_4)_2HPO_4$ 0.2 per cent, KH_2PO_4 0.3 per cent, NaCl 0.5 per cent, and $MgSO_4 \cdot 7H_2O$ 0.01 per cent, was used as the growing medium instead of broth.

The thick suspension of the cells in *M*/10 phsphate buffer (pH 7.0) was added with one mg. per ml. of the substrate and shaken in air for three hours at 27°. The washed suspensions were taken as the cells adapted to the substrate.

Manometry—Conventional Warburg manometric technique was used for the estimation of oxygen uptake and carbon dioxide output due to the oxidative degradation of the substrate at 30°, in air.

Main compartment: 1.0 ml. of bacterial suspensions and 0.8 ml. of *M*/10 phosphate buffer (pH 7.0)

Center well: 0.2 ml. of 20 per cent aqueous solution of potassium hydroxide

(omitted for the estimation of carbon dioxide),

Side arm: 0.2 ml. of *M*/100 substrate solution (substituted by water in control for endogeneous respiration of the cells).

For the rough estimation of ammonia production, 0.2 ml. of concentrated potassium hypobromite solution placed in the second side arm was added at a given time and the nitrogen gas evolution from ammonia, whose recovery laid between 93 to 97 per cent cent, was measured manometrically. All figures stated below were subtracted from those based on auto-respiration of the cells, whose Q_{O_2}, Q_{CO_2} and Q_{NH_3} were about 10~15, 8~13 and 2~5, respectively. All inhibitors were added to buffer previously, and their concentration was expressed as the final one.

Detection of Metabolic Intermediates—For a paper chromatographic analysis of diazo-positive compounds, reaction mixture in tenth-fold scale of the above was centrifuged from the cells and the concentrated supernatant was used as the sample without preliminary fractionation and the known compounds were run at the same time for comparison. Other technique was the same as that described previously by Miyaki *et al.* (*1*, *2*).

Volatile organic acids were converted to the hydrazides which were chromatographed by the ascending method with lutidine-isoamyl alcohol-water (2:7:1 *v/v*) and detected with ammoniacal silver nitrate. Steam distillate from the acidified supernant of the reaction mixture in fifty-fold scale of manometric experiments was neutralized with ammonia and concentrated to a syrup in vacuum. The residual salt was converted with some excess of ammonium rhodanate to an amide (*13*), and the alcoholic extract gave acylhydrazide by treatment with hydrazine hydrate, without appreciable loss of the original acid. The chromatographic technique itself was the same as that described previously by Satake and Seki (*14*).

Peroxide accumulated in the reaction mixture was detected by means of filter paper impregnated with lead sulfide as described by Kempf (*15*) and, acidic solution of ferric chloride and potassium ferricyanide by Schönbeim (*15*).

RESULTS AND DISCUSSION

Metabolic Path of Histamine—The results obtained by unadapted cells of *Achromobacter* grown on broth, with histamine, imidazole-acetic and -propionic acids are shown in Fig. 1. These substrates showed appreciable oxygen uptake after more or less lag periods, but in the presence of *M*/1000 streptomycin the oxidation scarcely occurred. In Fig. 2 are shown the results of the cells previously adapted to histamine on the substrates, which were oxidized immediately even in the presence of the antibiotic. These facts were assumed to indicate that the cells grown on broth had no enzyme systems for the oxidation but formed them successively (*16*) (or simultaneously) by the adaptation to histamine, and streptomycin, to which *Achromobacter* was very sensitive, inhibited

the adaptative enzyme formation (*16*) without any influence on the oxidative destruction itself.

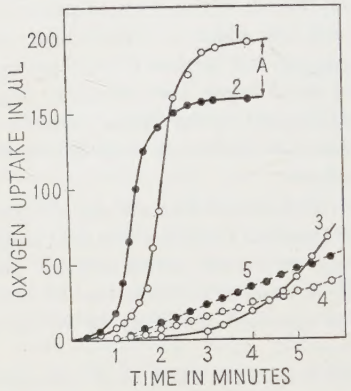


FIG. 1. Oxygen uptake by the cells grown on broth.

Added with (1) histamine, (2) imidazole-acetic acid, (3) imidazole-propionic acid, (4) histamine+*M*/1000 streptomycin, (5) imidazole-acetic acid+*M*/1000 streptomycin.

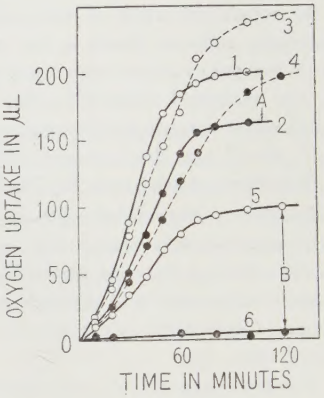


FIG. 2. Oxygen uptake by the cells previously adapted to histamine.

Added with (1) histamine, (2) imidazole-acetic acid, (3) histamine+*M*/1000 streptomycin, (4) imidazole-acetic acid +*M*/1000 streptomycin, (5) histamine+*M*/100 arsenite, (6) imidazole-acetic acid+*M*/100 arsenite.

When using the cells adapted to imidazole-acetic acid, histamine was not oxidized immediately but after about the same lag period as that with the unadapted cells, while in imidazole-acetic and -propionic acids the oxidation occurred immediately. In turn imidazole-acetic acid was oxidized immediately by the cells previously adapted to imidazole-propionic acid and the ratio of their rates of oxygen uptake due to the oxidation was almost the same both in cases, as shown in Fig. 3. Imidazole-4-carboxylic acid, -aldehyde, 4-hydroxymethyl-imidazole, urocanic acid and imidazole-lactic acid could not be oxidized immediately. These suggested an important key to the elucidation of the metabolic path of histamine.

As *M*/200 arsenite inhibited the oxidation of imidazole-acetic acid completely, it was used as the selective inhibitor on the destruction of histamine by the cells previously adapted to the amine, and appreciable accumulation of a diazo-positive substance in the reaction mixture was detected by paper chromatography. The R_f values with butano

saturated with water or 1 *N* ammonia, butanol-acetic acid-water (4:1:2 *v/v*), butanol-ethanol-water, 60 per cent hydrated pyridine or 70 per cent hydrated acetone agreed with those of synthetic imidazole-4-acetic acid. Some of the results are shown in Fig. 4. By means of quantitative paper chromatography (*12*), imidazole-acetic acid produced during one hour was found to be 60 to 75 per cent of the amine added and more than 90 per cent of the amine changed. During the oxidation 3.7–4.1 atoms of oxygen were taken up per one mole of the histamine added (shown as B in Fig. 2), while in the absence of the inhibitor the difference of oxygen uptake due to the oxidative degradation of histamine and of imidazole-acetic acid (shown as A in Figs. 1 and 2) was about 2 atoms, about onehalf of the former. Moreover, in the presence of *M*/200 arsenite, the bacterial catalase was found to be completely inhibited and an accumulation of hydrogen peroxide was detected during the restricted oxidation of histamine and only in the presence of cells, substrate, and the inhibitor.

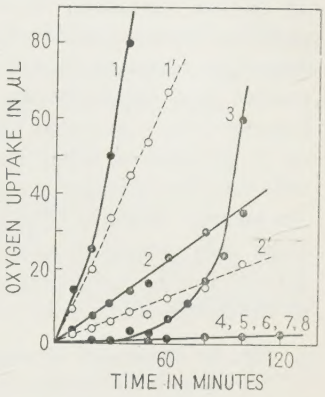
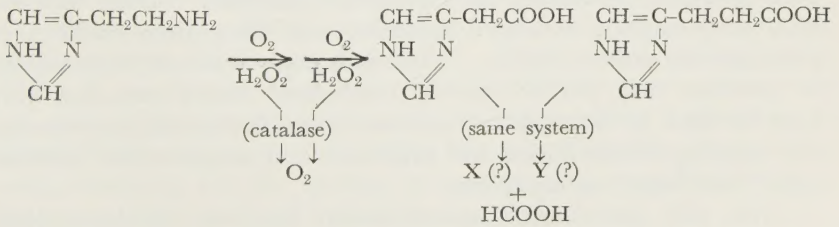


FIG. 3. Oxygen uptake by the cells previously adapted to imidazole-acetic acid (—•—•—) and imidazole-propionic acid (—o—o—).

Added with (1) imidazole-acetic acid, (2) imidazole-propionic acid, (3) histamine, (4) imidazole-carboxylic acid, (5) urocanic acid, (6) hydroxymethyl-imidazole, (7) imidazole-aldehyde and (8) imidazol-lactic acid.

The results of these experiments suggest that histamine was oxidized by an adaptive system of bacteria initially at the amino group of its side-chain, giving hydrogen peroxide (2 moles) and imidazole-4-acetic acid, the former being destroyed by the catalase and the latter by another adaptive system which acts on imidazole-propionic acid but not on urocanic acid, a metabolic intermediate of histidine.

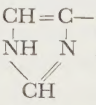


It follows, therefore, that the initial reaction just the same as that catalysed by mammalian diamine oxidase which is considered to convert histamine, consuming one mole of oxygen, to hydrogen peroxide, ammonia, and probably an aldehyde (*17*). Owing to the instability, however, the posturated aldehyde has not been confirmed as yet, same as in the case of other diamines (*18*). Imidazole-4-acetic acid is the only and first definite metabolic intermediate of histamine, as far as we are aware. Further path of this destruction is still obscure but acetone-dried cells grown on ammonium-glucose synthetic medium has far less activity for the oxidation of formic acid than those previously adapted to imidazole-acetic acid or histidine.

Q_{O_2} for formic acid of acetone-dried cells of *Achromobacter*

Grown on synthetic media:

No treatment	6—11
Adapted to histamine	40—45
Adapted to histidine	46—50
Grown on broth:	22—32

This might be interpreted as indicating the formation of formic acid from imidazole-acetic acid. The most probable precursor, if it were present, might be C_2 -atom of the imidazole similar to the conversion of urocanic acid to glutamic acid, though catalysed by a different system of the cells. The cleavage of the imidazole ring might occur, without further change at 4-position (*e.g.* oxidation or shortning) as R—CH_2-COOH and R—CH_2CH_2COOH, but not R—CH_2OH, R—CHO, R—COOH, R—CH=CH—COOH or R—CH_2—CH(OH)—COOH (where R is ) are destroyed by the adaptive system.

As already shown in Fig. 2, 7–8 atoms of oxygen were taken up during the oxidation of histamine, and 2.7–3.0 moles of carbon dioxide were produced, indicating an incomplete oxidation. Added with *M*/1000 streptomycin, however, histamine was completely oxidized to ammonia and carbon dioxide. These facts imply that with *Achromobacter* all products may not be entirely catabolized and a part may have been fed back to the anabolism as a result of sufficient supply of energy and building blocks during the oxidation, and streptomycin inhibited such ' feed back ' or anabolism.

Not only *Achromobacter* isolated freshly from the soil but various

laboratory strains of bacteria (isolated from putrefying food by Nakazima some years ago (*19*)) were found to form histamine metabolizing system adaptively as summarized in Table I and to oxidize the amine via imidazole-acetic acid with some ' feed back ' or anabolism.

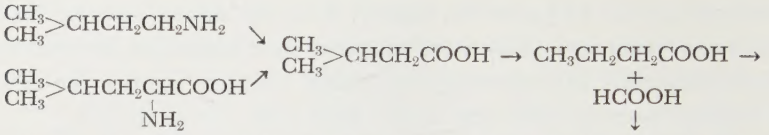
TABLE I

Oxidative Destruction of Histamine, Isoamylamine and Putrescine by Various Bacteria

Substrate / Bacteria	Histamine		Putrescine		Isoamylamine	
	(a) Q_{O_2}	(b) Lag period	(a) Q_{O_2}	(b) Lag period	(a) Q_{O_2}	(b) Lag period
		minutes		*minutes*		*minutes*
Serratia marcescens	30	300	40	210	0	—
Proteus vulgaris	75	200	70	170	50	180
Escherichia coli	0	—	20	300	0	—
Flavobacter fuscum	20	240	60	180	25	300
Bacillus mycoides	0	—	0	—	0	—
Pseudomonus Schuylkilliensis	60	230	45	190	80	150
Bacillus subtilis	120	170	95	170	40	230
Achromobacter liquidum	60	130	80	90	0	—
Bacillus mesentricus	40	250	70	180	0	—
Micrococcus subflavus	60	210	50	150	0	—

Oxygen uptake, subtracted from those based on endogeneous respiration, occured after a lag period (presented in (b)) and the rate increased to the maximum (shown in (a)). Bacterial cells were harvested from 24-hours broth culture at 27°.

Metabolic Path of Isoamylamine—With the cells of *Achromobacter* grown on broth, isoamylamine was oxidized after some lag period (60 minutes), but for the convenience of the successive adaptation technique (*16*) for the elucidation of the metabolic path, the cells grown on synthetic medium were used in this experiment. These results, summarized in Table II, are interpreted to mean that *Achromobacter* grown on a synthetic medium has no enzyme systems for the oxidative degradation of isoamylamine or leucine, but causes enzymic adaptation by a contact with substrates, and the systems, whose formation necessitates more or less lag period, catalysed following changes.



While the substrate postulated to be of lower metabolic order is oxidized immediately by the cells previously adapted to a compounds considered to be of higher order, the latter substrate are not oxidized immediately but after a lag period, by the cells unadapted or adapted to the former substrate. Oxygen uptake and carbon dioxide output of each postulated compound are also in agreement with the scheme given above.

TABLE II

Oxidative Destruction of Isoamylamine and Leucine by Achromobacter sp.

Substrates		(a) Oxygen uptake in initial 30 minutes						(b) Lag period	(b) O_2 : CO_2 : NH_3 (expressed in μl.)
		Cells adapted to					Cells unadapted		
		1	2	3	4	5			
								minutes	
Isoamylamine	(1)	95	<5	<5	<5	<5	<5	110	288 : 130 : 39
Isovaleric acid	(2)	78	85	<5	<5	71	<5	70	243 : 125 : —
Formic acid (*M*/300)	(3)	35	33	40	<5	30	<5	20	63 : — : —
n-Butyric acid	(4)	65	70	<5	81	68	<5	60	181 : 93 : —
Leucine	(5)	<5	<5	<5	<5	102	<5	150	274 : 176 : 37

Measured (a) in the presence and (b) absence of *M*/1000 streptomycin, which inhibited enzyme adaptation but not its oxidation. (for details see the text)

The initial path, the oxidative deamination to isovaleric acid was further elucidated. As *M*/1000 8-hydroxyquinoline completely inhibited the oxidation of isovaleric acid, using it as the selective inhibitor on the destruction of isoamylamine by the cells previously adapted to the amine, appreciable accumulation of isovaleric acid and hydrogen peroxide were detected. The acid was isolated as S-benzylthiouranium salt in 63 per cent yield, together with isovaleraldehyde (as dimedone derivative) in 8 per cent yield, based on the added amine respectively.

Using *M*/1000 $\alpha\alpha'$-bipyridyl instead of 8-hydroxyquinoline, butyric acid was detected, though in a very small amount, together with a large amount of isovaleric acid as shown in Fig. 5. In manometric

TABLE III

Isolation of Metabolic Intermediate of Isoamylamine

One g. of the cells previously adapted to isoamylamine, 1500 mg. of isoamylamine hydrochloride, 1000 ml. of *M*/10 phosphate buffer (pH 7.0) containing *M*/1000 8-hydroxyquinoline

↓ each 100 ml. of the above mixture placed in a Sakaguchi flask and shaken at 27° for 5 hours

↓ centrifuged

Combined supernatant

↓ steam distillation

Residue	Distillate
↓ acidified and steam distilled	↓ extracted with ether
Distillate	Ether layer
↓ neutralized with bicarbonate, concentrated and added with equivalent amount of S-benzyl-thiouranium chloride (1.7 g.)	↓ solvent removed in vucuum, 10 ml. water added, 500 mg. of alcoholic solution of dimedone added slowly
S-Benzylthiouranium isovalerate	Crystals
↓ recrystallized from hydrated alcohol	↓ recrystallized from hydrated alcohol
Melted at 153.0° and showed no depression with the authentic specimen. Yield, 2060 mg. (63%)	Melted at 156–7° and showed no depression with authentic dimedone derivative of isovaleraldehyde. Yield, Yield, 204 mg. (8%)

experiments on the restricted oxidation of isoamylamine in the presence of 8-hydroxyquinoline, 3.4-3.9 atoms of oxygen were consumed without unchanged amine, while in the absence of the inhibitor the difference of oxygen uptake of the amine and of isovaleric acid was 1.9–2.1 atoms, one-half of the former case. Moreover, in the presence of *M*/1000 8-hydroxyquinoline, bacterial catalase was found to be inhibited completely.

These results have shown that isoamylamine is also oxidized to isovaleric acid just in the same way as histamine, as follows:

$$\text{R-CH}_2\text{NH}_2 \xrightarrow[\text{H}_2\text{O}_2]{\text{O}_2} \text{NH}_3 + \text{R-CHO} \xrightarrow[\text{H}_2\text{O}_2]{\text{O}_2} \text{R-COOH}$$

(Catalase)

↓

reproduction of O_2.

The acid, which is also a metabolic intermediate of leucine with *Achromobacter*, seems to be demethylated oxidatively to butyric acid and formic

acid. The process is still obscure but the accumulation of butyric acid together with isovaleric acid, in bacterial culture containing leucine has been reported by several workers (*20*).

Results obtained with the cells of other bacteria are shown in Table I.

Metabolic Path of Putrescine—The results of experiment obtained with *Achromobacter* grown on broth in the presence of putrescine and γ-aminobutyric acid are summarized in Table IV, which indicate that putrescine is oxidized *via* γ-aminobutyric acid by an adapted system of the cells. Results obtained with other bacteria are shown in Table I.

TABLE IV

Oxidative Destruction of Putrescine by Achromobacter sp.

Substrate		(a) Oxygen uptake in initial 30 minutes			(b) Lag period
		Cells adapted to		Cells unadapted	
		(1)	(2)		
					minutes
Putrescine	(1)	101	<5	<5	50
γ-Aminobutyric acid	(2)	85	96	<5	40

Measured in the presence (a) and absence (b) of *M*/1000 streptomycin.

Interrelation of Bacterial Amine-oxidizing Systems—The three amines tested above, were oxidized at first to the corresponding carboxylic acids, probably in the same manner as postulated by Zeller (*5*) and Werle (*6*). Nevertheless, the cells previously adapted to an amine did not oxidize other amines immediately but after a lag period, as shown in Table V. With *Achromobacter*, the adaptive substrate, which was different from the one used for the enzyme adaptation but oxidizable by the adaptive system, seemed not to necessitate an apparent lag period (more than 5 minutes) for the penetration into the cells, as imidazole-acetic (or -propionic) acid was found to be oxidized immediately with the cells previously adapted to imidazole-propionic (or -acetic) acid. These results, therefore, might be interpreted to mean that the adaptive systems for each amine are not strictly the same, though their reaction type and behaviors (as mentioned below) are very similar to each other.

This conclusion has been further verified by the result of following simultaneous adaptation. The cells grown on bouillon were suspended in *M*/10 phosphate buffer (pH 7.0) containing two adaptive substrates,

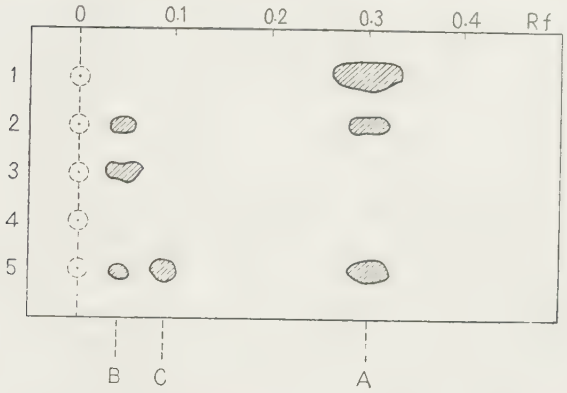


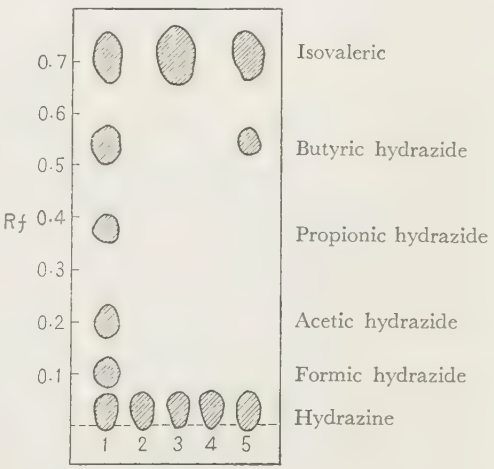
FIG. 4. Paper chromatographic analysis of restricted oxidative products of histamine with *Achromobacter sp.* in the presence of *M*/200 arsenite.

(1) Before reaction, (2) after one hour, (3) after two hours, (4) same as (3) except omission of histamine from the complete system (for details see the text), (5) synthetic mixture of histamine (A), imidazole-acetic (B) and -propionic acids (C) for comparison.

These were ran with butanol (saturated with *N* ammonia) and colored with diazobenzenesulfonic acid and alkali.

FIG. 5. Paper chromatogram of restricted oxidative products of isoamylamine by *Achromobacter* in the presence of selective inhibitors. (1) Synthetic mixture of formic, acetic, propionic, butyric and isovaleric hydrazides as control. (2) Before and (3) after reaction in the presence of *M*/1000 8-hydroxyquinoline. (4) Before and (5) after reaction in the presence of *M*/1500 $\alpha\alpha'$-bipyridyl

(for details, see the Experimental Parts)



histamine and putrescine, and shaken at 27° in air. At a given time the cells were centrifuged, washed and the activities of both substrates

were measured in the presence of $M/1000$ streptomycin to prevent further enzyme adaptation during the measurment of activities.

Simultaneous adaptations to both substrates were observed at different rates and with different lag periods as shown in Fig. 6. If both deaminations were catalyzed by the same system, above fact cannot be explained.

Gale (*7*) reported that putrescine seemed to be oxidized by the constitutive enzyme systems of *B. coli* and *Pseudomonus*. This might have been due to the amine present or produced in the growing media and the cells adapted to it during their growth. Zeller (*5*) and Werle (*6*) may also have used cells adapted to various amines simultaniously and concluded that diamines and histamine were oxidized by the same enzyme, though we are unable to know their experiments in detail.

TABLE V

Oxidation of Various Amines by Achromobacter sp. Grown on Broth

Substrate		Cells adapted to				Unadapted cells	
		(1)	(2)	(3)	(4)		Lag period
							minutes
Histamine	(1)	++	+	+	+	+	70
Putrescine	(2)	+	++	+	+	+	50
Cadaverine		−	−	−	−	−	<300
Isoamylamine	(3)	+	+	++	+	+	60
β-Phenethylamine	(4)	+	+	+	++	+	80
Tyramine		−	−	−	−	+	<300

++: Oxygen uptake occured immediately.
+: After lag period.
−: No oxygen uptake.

Some Properties of the Bacterial Amine Oxidizing Systems—Though the oxidizing systems for each amine to the corresponding carboxylic acid are not the same, their properties seem to be very similar. As shown in Fig. 7, oxygen uptake due to the destruction of each amine by the cells previously adapted, was found over a wide range of pH and their activity-pH curves were similar to that of mammalian diamine oxidase. The same cells grown on bouillon oxidized leucine in a much smaller range of pH, so that the wideness of pH range should not be concluded only from the measurment of overall oxygen uptake by a rather complex

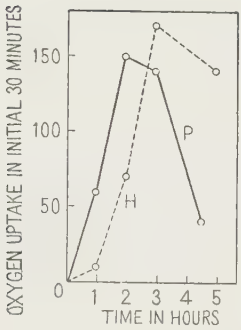


FIG. 6. Simultaneous adaptation to putrescine (P) and histamine (H).

Abscissa: Period of contact with substrates

Ordinate: Activities for both substrates (for detail see the text)

Activity of adaptive systems decreased rapidly after consumption of the substrate with some lag period.

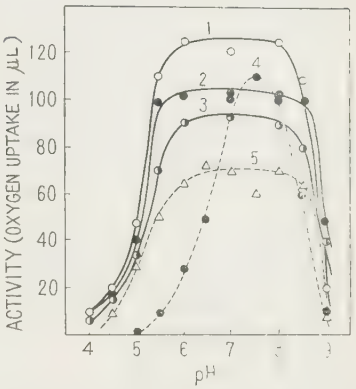


FIG. 7. pH-Activity of bacterial oxidation of amines. Bacterial oxidation of (1) isoamylamine, (2) putrescine, (3) histamine, and (4) leucine. (5) Mammalian diamine oxidase (putrescine) as control. *M*/20 phthalate-phosphate-borate buffer was used in these experiments. The activity is expressed as oxygen uptake in initial 30 minutes.

enzyme system in the cells. Miyaki *et al.* (*1*) had already found that the destruction of histamine in putrefying food could occur even in an acidic range (pH 5.0–5.5).

Representative figures for influences caused by various compounds on the bacterial oxidation of histamine, isoamylamine and putrescine are listed in Table VI. The substrate concentration could be varied widely (*M*/2000–*M*/100) with very little change, if any in the rate of oxygen uptake and the inhibitory power. The wide range of optimal pH and the effect of various compounds, especially the powerfull inhibition by cyanide, methylene blue and carbonyl reagents, and slight effect of azide, fluoride, iodoacetate, arsenite and metal reagents, were similar to the case of mammalian diamine oxidase (*27*). It must be emphasized that the bacterial deamination of isoamylamine was inhibited by the carbonyl reagents, which did not affect the deamination by mammalian monoamine oxidase (*24*, *25*) (no effect even at *M*/500). Powerful inhibition by heavy metal cations might have resulted from the use of intact cells. S-Alkylthioureas, well-known inhibitors which compete with the substrate on mammalian amine oxidases (*22*),

also inhibited those bacterial deamination, though the effect could not be altered by the change of the substrate concentration in this case.

TABLE VI

Inhibition of Bacterial Oxidation of Amines

Inhibitor (final concentration)	Inhibition of oxidative destruction of		
	Histamine	Putrescine	Isoamylamine
	per cent	*per cent*	*per cent*
M/1000 Hydroxylamine	100	100	100
" Hydrazine	100	70	100
" Semicarbazide	100	50	100
M/300 Dimedone	<10	<10	<10
M/1000 Potassium cyanide	100	100	100
" Methylene blue	100	100	100
M/3000 " "	60	55	65
M/200 Methyl orange	0	0	0
M/100 S-Benzyl thiourea	80	65	100
" S-Methyl "	30	20	50
" Arcaine	0	0	0
M/500 Guanidine	0	0	0
M/100 Sodium azide *	<10	<10	<10
" Sodium fluoride *	<10	<10	<10
M/300 Iodoacetate *	<10	<10	<10
M/1000 Ag^{+}, Cu^{++}, Hg^{++}	100	100	100
M/200 Arsenite *	30	<10	<10
M/1000 $\alpha\alpha'$-Bipyridyl *	20	10	20
M/1000 8-Hydroxyquinoline*	80	15	35

* Enzymatic formation completely inhibited.

Inhibition by methylene blue seems to be a specific one for amine oxidases, including mammalian mono- and di-amine oxidases and the bacterial ones. Egami *et al.* (*24*, *25*) considered the effect of methylene blue due to its 3,6-dimethylamino groups and showed that mammalian monoamine oxidase was inhibited by the dye and even by dimethylaniline, but far less by phenothiazine itself. In the bacterial deamination of isoamylamine, however, aromatic dimethylamino compounds such as methyl orange (*p*-dimethylamino-*p'*-azobenzenesulfonic acid) and aliphatic ones, such as anergen (NN-dimethyl-N'-benzyl-N'-phenyl-

TABLE VII

Inhibition by Antihistamine and Antiadrenalines

Compound	Deamination of		
	Isoamylamine	Putrescine	Histamine
$C_6H_5CH_2$, R, NCH_2CH_2N, CH_3, CH_3	no effect	n=2	n=4
R: Phenyl- (Antergan)	at 2.0×10^{-3} *M*	$\Phi=0.96\times10^{-3}$ *M*	$\Phi=0.69\times10^{-2}$ *M*
α-Pyridyl- (Pyribenzamine)		1.12 „	0.95 „
2-Thiazol-		1.32 „	1.05 „
R′, NCH_2CH_2Cl, R	100% inhibition	n=4	n=2
R: β-$C_{10}H_7CH_2$-, R′: C_2H_5—	at 0.1×10^{-3} *M*	$\Phi=0.34\times10^{-3}$ *M*	slightly inhibition
R=R′: $C_6H_5CH_2$-		0.26 „	at 2.0×10^{-3} *M*
Phenothiazine	n=4	n=4	n=4
3,6-Dimethylamino-(Methylene blue)	$\Phi=0.33\times10^{-3}$ *M*	$\Phi=0.33\times10^{-3}$ *M*	$\Phi=0.33\times10^{-3}$ *M*
10-β-Dimethylaminoethyl- (Anergen)	0.60 „	0.35 „	0.29 „
10-β-Dimethylamino-isopropyl- (Phenargen)	0.41 „	0.34 „	0.22 „
Anergen chloromethylate	0.74 „	0.41 „	0.35 „

Remarks: $H=\frac{[G]^n}{[\Phi]^n+[G]^n}$, where H is the degree of inhibition, G is the concentration of inhibitor, and n and Φ are constant. Φ correspnds to the concentration of the inhibitor to give a 50 per cent inhibition.

ethylenediamine) and benadryl were found to give no effect, while phenothiazine derivatives, such as anergen and phenargen, though having aliphatic diamino instead of aromatic ones, were most powerful inhibitors. It should, therefore, be concluded that at least in the bacterial deamination the phenothiazine ring, whose structure resembles that of flavine, assumed to be coenzyme of amine oxidases, plays an important rôle in inhibiting the deamination.

Contrary to the above-mentioned effects, inhibition by antihistamine and antiadrenaline drugs showed a dramatic difference between the bacterial deamination of histamine and isoamylamine. As a whole, antihistamines of ethylenediamine type inhibit the oxydation of histamine but do not inhibit that of isoamylamine at all. While antia-

adrenaline of β-chloroethyl-monoamine type, which antagonized some monoamine as noradrenaline in vivo, specifically antagonized the oxidation of isoamylamine. Thus, some parallelism between a pharmacological activity and enzyme inhibition can observed. Phenothiazine type, as mentioned already, are powerful inhibitors for all the deamination but anergen or phenargen, having strong antihistaminic properties, seem to inhibit the deamination of histamine far stronger than that of other amines, which are effected at the same or rather less degree than methylene blue. The discussion on these results will be published elsewhere together with the effects of these durgs on mammalian amine oxidases.

SUMMARY

1. *Achromobacter* grown on broth was able to form adaptive system to oxidize histamine, isoamylamine and putrescine.

2. Histamine was metabolized *via* imidazole-acetic acid. The acid was destroyed by a system acting also upon imidazole-propionic acid, giving formic acid.

3. Isoamylamine was oxidized via isovaleric acid, a metablic intermediate of leucine, by the same bateria. The acid seemed to be demethylated to formic and butyric acids.

4. Differing from mammalian amine oxidases, these bacterial systems were strictly specific for each amine, though their behaviors were very similar to those of mammalian diamine oxidase.

5. Antihistamine drugs inhibited the bacterial oxidation of histamine, but not of monoamine, while antiadrenaline inhibited that of isoamylamine alone.

6. These results were discussed.

We wish to express our sincere thanks to Prof. S. Akabori and Prof. K. Miyaki for their interest in this study. We also extend our thanks to Mr. M. Hayashi for his discussion of this study, to Prof. K. Aiso for identification of bacteria, to Dr. S. Yamaoka, Mr. S. Seno and Mr. S. Sakurai for the sample offered.

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BILE ACID OF THE ALLO-SERIES

II. SYNTHESIS OF 12-HYDROXY-7-KETO-ALLOCHOLANIC ACID AND ITS DERIVATIVES

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When 3-keto-4-bromo compound of bile acid nucleus was dehydrobrominated and then reduced, the cholane nucleus could be partially rearranged into an allocholane nucleus. By this method, we synthesized 3,β-12α-dihydroxy-allocholanic acid (allodesoxycholic acid) (*1*) and 3,β-7,12-trihydroxy-allocholanic acid (β-cholic acid) (*2*). Bromination of 7-keto-cholestanyl acetate proceeded less readily (*3*). In sterol with cholane nucleus, the character of 6-bromo compound was not clear and it was very difficult to substitute the hydrogen atom of the 6-position with bromine atom. Only in the 7-keto compound which had no substitution group in the A ring of the cholane nucleus, 6-bromo-7-ketonic acid was isolated in a stable form (*4*). But in the 3-hydroxy-7-ketocholanic acid, the brominated material of the 6-position became a resin-like substance due to the disturbance of stereo-configuration and so could not be isolated.

The 3-keto-7,12-dihydroxycholanic acid was reduced into the 7,12-dihydroxycholanic acid by the Clemmensen reduction. This acid was partially oxidized into 12-hydroxy-7-ketocholanic acid by $KMnO_4$. Brominated 12-hydroxy-7-ketocholanic acid did not crystallize, but a stable 6-bromo compound was isolated. The 12-hydroxy-7-keto-6-bromo compound was dehydrobrominated with a mixed solution of pyridine and silver nitrate to produce Δ^5-7-keto-12-hydroxycholenic acid (m.p. 204°). The absorption maximum of this acid was α^{alc}_{max} 238 mμ, 320 mμ. This value was similar to that of the acid which Baba (*5*) obtained from 3-acetoxy-6-bromo-7-ketocholanic acid. When Δ^5-7-keto-12-hydroxycholenic acid was reduced with hydrogen, 7-keto-12-hydroxy-allocholanic acid (m.p. 199°) was obtained. It was oxidized into 7,12-diketo-allocholanic acid (m.p. 187.5–189°).

EXPERIMENTAL

7-Keto-12-hydroxycholanic Acid—By the Tai Sihk Sihn's method (*6*), 3-keto-7, 12-dihydroxycholanic acid (m.p. 181°) was prepared and 40 g. of this acid were dissolved in a mixed solution of 120 ml. of methanol and 40 ml. of toluol. Then 20 g. of amalgamated zinc were added and the mixture was refluxed for 3 hours on a sand bath. To the mixed solution 40 ml. of methanol and 50 ml. of concentrated HCl were added in three times while heating. After reduction, the toluol part was separated from the water part and the water part was extracted with ether and the ether extract was added to the toluol part. The precipitate, which was obtained after the toluol and ether had been evaporated in vacuum, was recrystallized from alcohol to obtain methyl 7,12-dihydroxycholanate (m.p. 170–171.5°). The acid which was hydrolysed with 10 per cent potassium hydroxide, was recrystallized from alcohol or methanol. 25 g. of 7,12-dihydroxycholanic acid (m.p. 203–205.5°) were obtained. In 1 liter of 2 per cent Na_2HPO_4 solution, 1 g. of 7,12-dihydroxycholanic acid was dissolved and an aqueous solution of 0.4 g. $KMnO_4$ was added to it. The mixture was kept at room temperature for 40 hours (1.3 moles or 1.5 moles of $KMnO_4$ showed similar reaction).

After this treatment, a dark brown precipitate of manganese dioxide appeared. This precipitate was filtered off and washed with small volume of water. When the filtrate was acidified with diluted HCl, a precipitate came out. This precipitate was extracted with a large quantity of ether and the ether part was washed with water and dried. Upon evaporating ether, a crystal was obtained. When the crystal was recrystallized from benzol, 0.8 g. of a rectangular crystal of 7-keto-12-hydroxycholanic acid, m.p. 176° was obtained. This crystal coincided with the substance which had been reported previously (*7*).

Δ^5-7-Keto-12-hydroxycholenic Acid—0.533 g. of 7-keto-12-hydroxycholanic acid was dissolved in 20 ml. of glacial acetic acid and to it were added 5 ml. of glacial acetic acid containing 1.2 g. of bromine and a few drops of HBr. This was heated for a few minutes on a water bath. After decolorization of bromine color, this solution was poured into ice water, and then filtered. The solution was chromatographed, and the elution was performed with ether, but no crystals were obtained. 0.15 g. of 7-keto-6-bromo-12-hydroxycholanic acid was dissolved in 5 ml. of pyridine and to it was added 0.525 g. of silver nitrate. This was heated for 12 hours (with reflux) on a sand bath. After operation, this solution was poured into a large quantity of water, acidified with dilute HCl to produce a precipitate. This precipitate was extracted with ether and then the ether was evaporated. The residue was recrystallized from ethyl acetate to make a column-like crystal of Δ^5-7-keto-12-hydroxycholenic acid (m.p. 204°). Beilstein's reaction of this substance was negative, and its absorption maximum was α^{alc}_{max} 238 mμ. 320 mμ.

Analysis. Calcd. for $C_{24}H_{36}C_4$: C 74.23; H 9.28
Found: C 74.08; H 9.63

7, 12-Diketo-allocholanic Acid and Its Derivative—0.1 g. of Δ^5-7-keto-12-hydroxycholenic acid was dissolved into 6 ml. of glacial acetic acid and reduced with 1 mole of hydrogen

and 0.015 g. of Adam's platinium oxide. The reaction solution was poured into a large quantity of water, and the appeared precipitate was extracted with ether. After evaporation of ether, this was recrystallized from methanol. The saturated acid of 7-keto-12-hydroxy-allocholanic acid (m.p. 199°) was obtained as needle-like crystals. Methylester of this acid was recrystallized from acetone water; m.p. 158–159.5°. 0.2 g. of the methyl 7-keto-12-hydroxy-allocholanate was dissolved into 3 ml. of glacial acetic acid and to this was added 2 ml. of glacial acetic acid which contained 66 mg. of chromic anhydride. This was kept for 1.5 hours at room temperature. The precipitate, which appeared by the addition of water, was extracted with ether. The ether-soluble fraction was recrystallized from acetone water to produce methyl 7,12-diketo-allocholanate with m.p. 122–123°.

Analysis. Calcd. for $C_{25}H_{38}O_4$: C 74.63; H 9.45
Found: C 74.54; H 9.70

The above methyl 7,12-diketo-allocholanate was hydrolysed, and the obtained acid was recrystallized from acetone water, producing 7,12-diketo-allocholanic acid (m.p. 187.5–189°).

Analysis. Calcd. for $C_{24}H_{36}O_4 \cdot H_2O$: C 70.94; H 9.36
Found: C 71.03; H 9.33

To 0.47 g. of 7,12-diketo-allocholanic acid 1 g. of 60 per cent hydrazine hydrate and 15 ml. of triethylen glycol were added and then heated with reflux for one hour. Following 1 g. of NaOH was added and kept at 180–200° for 1.5 hours. After the reaction was over, this mixed solution was poured into water and acidified with HCl. The precipitate thus produced was extracted with ether and the ethereal extract was washed with water and dried. The residue obtained by evaporating ether was dissolved into a mixture of 20 g. of *n*-propyl alcohol and 1 g. of concentrated H_2SO_4, and the solution was heated on a water bath for 2.5 hours. When water was added to this reaction mixture, a precipitate was produced. This was extracted with ether and evaporated. The residue was recrystallized from methanol to produce needle-like crystals (m.p. 101–102.5°). The melting point was not lowered, when the crystals were mixed with pure *n*-propyl allocholanate.

Analysis. Calcd. for $C_{27}H_{46}O_2$: C 80.59; H 11.44
Found: C 80.01; H 11.62

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BILE ACID OF THE ALLO-SERIES

III. ON THE SYNTHESIS OF $\Delta^{3,5}$-7-KETO-12-HYDROXYCHOLADIENIC ACID AND ITS DERIVATIVES

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Sasaki and Mochizuki (*1*) synthesized 7-keto-12-hydroxyallocholanic acid by bromination of 7-keto-12-hydroxycholanic acid.

The author found that the 3-hydroxy-7-keto compound was brominated securely by protection of the hydroxyl group of the 3-position, and ethyl 3-acetoxy-6-bromo-7-keto-12-hydroxycholanate was obtained in stable form by bromination of the ethyl 3-acetoxy-7-keto-12-hydroxycholanate, although it was not crystal.

After this bromo compound was dehydrobrominated with silver nitrate in pyridine, it was hydrolyzed so as not to produce 3-hydroxy-Δ^5-7-keto-12-hydroxycholenic acid but to produce $\Delta^{3,5}$-7-keto-12-hydroxy choladienic acid (m.p. 205°). From this result it was noticed that the acetoxyl group of 3-position of the cholane nucleus was dehydrated. In detail: as soon as the double bond was produced at 5～6-position of cholane nucleus by hydrobromination with pyridine, the acetoxyl group of the 3-position was dehydrated and the second double bond was performed and thus changed to α, β, γ-unsaturated ketone. Such a reaction was as yet unknown in bile acid series. But possibly A. Lardon (*2*) obtained the same compound as $\Delta^{3,5}$-7-keto-12-hydroxy-etiocholadienic acid by dehydrobromination of methyl 3,12-diacetoxy-6-bromo-7-ketoetiocholanate. Another case was production of the double bond at the 3-position, when Clemmensen reduction was applied to dehydrocholic acid (*3*). Formerly, methyl hyodesoxycholate was subjected to dehydration by means of phosphorus oxychloride in pyridine, and afforded 3β-chloro-Δ^5-cholenate (*4*).

In the allo-series, Barr, Heilbron, *et al.* (*5*) obtained both 7-ketocholesteryl acetate and Δ^3-7-ketocholesterylene due to dehydrobromination of the bromo compound of the 7-ketocholestanyl acetate. These experiments showed that an important difference occurred in

attitude regarding the dehydrobromination of the 6-bromo compound by the configuration of the A:B ring of the normal and the allo-series.

In regard to dehydration of the acetoxyl or hydroxyl group of the 3-position, first of all a double bond occurred at the 5∼6-position by dehydrobromination of 7-keto-6-bromo compound. As a result Δ^5-α, β-unsaturated ketone was produced. It was thought that the dehydration of the acetoxyl or hydroxyl group at the 3-position was due to participation of the double bond of the 5∼6-position through formation of a hybrid ion intermediate. In a similar case, Louis F. Fieser *et al.* (*6*) reported that the methyl 3,7-diacetoxy-12-ketocholanate was converted with selenium dioxide into 3,7-diacetoxy-12-keto-$\Delta^{9(11)}$-cholenate, which on treatment with alkali afforded 3-hydroxy-12-keto-$\Delta^{7,9(11)}$-choladienic acid.

By hydrolysis of ethyl 3-acetoxy-Δ^5-7-keto-12-hydroxycholenate, it is questionable whether a similar dehydration occurred or not at the 3-position producing $\Delta^{3,5}$-7-keto-12-hydroxycholadienic acid.

On the free state of the hydroxyl group at the 3-position, it is unclear whether dehydration was difficult at the 3-position or was easy at only the acetoxyl group, because the 3-hydroxy-7-keto acid could not be brominated. Formerly, Kazuno and Baba (*7*) stated that in spite of acetoxyl group of the 7-position being dehydrated at the same time into the 3-keto-$\Delta^{4,7}$-12-acetoxycholadienic acid when 3-keto-4-bromo-7, 12-diacetoxycholanic acid was dehydrobrominated with pyridine, the 3-keto-4-bromo-7,12-dihydroxycholanic acid was changed into the 3-keto-Δ^4-7,12-dihydroxycholenic acid without dehydration.

Marker *et al.* (*8*) found that the 7-ketocholesteryl chloride, in which polarization of the double bond at 5∼6-position was in opposite direction to that required for interaction with 3-position, was converted by acetate ion into Δ^3-7-ketocholesterylene and 7-ketocholesteryl acetate that furnishes epicholesterol on Wolff-Kishner reduction.

Next, $\Delta^{3,5}$-7-keto-12-hydroxycholadienic acid was hydrogenated with palladium catalyst into Δ^5-7-keto-12-hydroxycholenic acid (m.p. 204°). It was the same compound as the Δ^5-7-keto-12-hydroxycholenic acid, which Sasaki *et al.* (*1*) derived from the 7-keto-12-hydroxycholanic acid. So this acid was converted into Δ^5-12-hydroxycholenic acid (m.p. 171—172°) through Wolff-Kishner reduction.

Then it was oxidized into Δ^5-12-ketocholenic acid (m.p. 215—218°), from which the methyl Δ^5-cholenate (m.p. 82°) was obtained through Wolff-Kishner reduction.

EXPERIMENTAL

Ethyl 3,12-*Dihydroxy*-7-*ketocholanate*—10 g. of cholic acid was dissolved in 10 per cent Na_2HPO_4 solution and diluted to 600 ml. with water and 2.6 g. of $KMnO_4$ in 200 ml. of water was added. After being kept at room temperature for about twenty-four hours, the solution was filtered. The filtrate was acidified with dilute HCl to make a precipitate, which was filtered and dried. It was boiled in ethanol which contained concentrated H_2SO_4 in the concentration of 7 per cent for five hours with reflux. The solution was poured into water to make a precipitate, which was extracted with ether and then washed with Na_2CO_3 and water, and dried over anhydrous Na_2SO_4. After the ether was distilled off, a crystalline residue was obtained. It was recrystallized from methanol to produce prisms or glistening leaflets (m.p. 160°).

Ethyl 3-*Acetoxy*-7-*keto*-12-*hydroxycholanate*—According to the Haslewood's method (*9*), a solution of ethyl 3,12-dihydroxy-7-ketocholanate (25 g.) in dry benzene (100 ml.) and pyridine (25 ml.) was cooled in ice and treated gradually, with shaking, with a 2:1 (*v/v*) mixture (50 ml.) of dry benzene and acetyl chloride. After three hours at 0°, the mixture was diluted with water, and extracted with ether. The extract, washed with dilute HCl and water, and dried over anhydrous Na_2SO_4, was evaporated and the residue was recrystallized from benzene and petroleum ether. The ester formed: glistening leaflets; m.p. 147–148°; yield 22 g.

Ethyl 3-*Acetoxy*-6-*bromo*-7-*keto*-12-*hydroxycholanate*—Ethyl 3-acetoxy-7-keto-12-hydroxycholanate (8.167 g.) was dissolved in glacial acetic acid (40 ml.) and a solution of bromine (2.76 g.) in glacial acetic acid (15 ml.) and five drops of HBr (sp. gr. 1.46) was added. The flask was stoppered and heated to 70°. As soon as the color of bromine vanished, the solution was poured into water to make a precipitate. It was filtered, extracted with ether, washed with water and dried. The solution was chromatographed on Al_2O_3 and the elution was performed with ether. Ether was distilled off, and the residue was dried under diminished pressure. In this way amorphous residue (9 g.) was obtained.

$\Delta^{3,5}$-7-*Keto*-12-*hydroxycholadienic Acid*—The bromo compound (9 g.) was dissolved in dried pyridine (80 ml.) and silver nitrate (25 g.) was added. The mixture was boiled on a sand bath with reflux for twenty four hours and cooled. The solution was poured into dilute HNO_3 to produce a precipitate, which was extractedwith ether, washed with water and dried over anhydrous Na_2SO_4. The ether solution was chromatographed on Al_2O_3 and from the eluate, ether was distilled off. The gummy residue was boiled with reflux for 20 minutes with 5 per cent methanolic NaOH, and the obtained solution was diluted with water. Methanol was distilled off under diminished pressure and acidified with HCl to produce a precipitate, which was filtered and dried. The dried precipitate was crystallized from ethanol as a powdery crystal. Recrystallization from ethanol and then ethyl acetate gave prisms, m.p. 205°, yield 2.5 g.

Analysis. Calcd. for $C_{24}H_{34}O_4$: C 74.61; H 8.80
Found: C 74.41; H 9.07

Δ^5-7-*Keto*-12-*hydroxycholenic Acid*—The above dienic acid (1 g.) was dissolved in ethanol (40 ml.), palladium black (200 mg.) was added to the solution, and partial catalytic

hydrogenation with hydrogen for 40 minutes was performed at room temperature (22°). The solution was filtered and evaporated to isolate a crystal, which was recrystallized, and thus a prism-like crystal (m.p. 204°) was obtained almost quantitatively. The double bond at 5∼6-position of this acid was somewhat inactive and the consumption velocity of bromine became slower.

Analysis. Calcd. for $C_{24}H_{36}O_4$: C 74.23; H 9.28
Found: C 74.76; H 9.44

Δ^5-12-*Hydroxycholenic Acid*—According to a Wolff-Kishner's method modified by Huang-Minlon (*10*), a mixture of Δ^5-7-keto-12-hydroxycholenic acid (0.6 g.), triethylene glycol (10 ml.), KOH (1 g.) and hydrazine hydrate (3 g.) was refluxed for half an hour at 165° on an oil bath. Then the reflux condenser was removed and the temperature of the solution was elevated to 200° for 2 hours. After cooling, the reaction mixture was diluted with water, acidified with dilute HCl. The produced precipitate was filtered and dried. This acid was esterified with ethereal diazomethane, and chromatographed on Al_2O_3. From the eluate, ether was distilled off, and the residue was hydrolyzed with 5 per cent methanolic NaOH to produce column-like crystals with m.p. 171–172°. The crystal showed negative Hammarsten reaction (yellow) and positive Ehrlich's aldehyde reaction (violet).

Analysis. Calcd. for $C_{24}H_{38}O_4$: C 77.01; H 10.16
Found: C 77.34; H 10.09

Δ^5-12-*Ketocholenic Acid*—$\Delta_2$12--Hydroxycholenic acid (0.32 g.) was oxidized with CrO_3 in glacial acetic acid in the usual way. To the reaction solution water was added and a lamellar crystal was isolated afterwards. Recrystallization from acetone and then ethanol gave lamellar crystal, m.p. 215–218°, yield 0.15 g.

Analysis. Calcd. for $C_{24}H_{36}O_3$: C 77.42; H 9.68
Found: C 77.60; H 9.80

Methyl Δ^5-*Cholenate Acid*—A mixture of Δ^5-12-ketocholenic acid (0.1 g.), triethylene glycol (5 ml.), NaOH (0.5 g.) and hydrazine hydrate (0.5 g.) was heated with reflux for half an hour at 160° and then kept at 200°. After cooling, the reaction solution was poured into water, acidified with dilute HCl and extracted with ether. The ether extract was esterified with diazomethane and chromatographed on Al_2O_3. After distilling off ether from the eluate, methyl Δ^5-cholenate was recrystallized from methanol to obtain 0.04 g. of needle-like crystals, m.p. 82°.

Analysis. Calcd. for $C_{25}H_{40}O_2$: C 80.65; H 10.75
Found: C 80.49; H 10.96

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EFFECTS OF SALT CONCENTRATION ON THE RESPIRATION OF A HALOTOLERANT BACTERIUM

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There are many bacteriological studies on halotolerant and halophilic bacteria (*1*, *2*, *3*, *4*, *5*, *6*), but few of them have been treated from a biochemical standpoint. Recently J. Robinson *et al.* (*7*, *8*, *9*) have reported on the mechanism of halophilism of *Micrococcus halodenitrificans* and the denitrification by the said organism. Independently, we have also been investigating the halotolerancy and the respiration of the halotolerant bacteria, and some of the results are reported in the present paper.

EXPERIMENTAL

Bacterial Suspension—A halotolerant bacterium isolated by H. Yamada and identified by S. Hosoya and M. Soeda as *Bacillus pumilus var.* was cultured on agar plate of peptone broth, pH 7.2, containing 10 per cent NaCl and 1 per cent KNO_3 at 37° for 24 hours. Bacterial cells were washed several times with a 10 per cent NaCl solution and finally suspended in a 10 per cent NaCl solution in every case unless otherwise stated.

Respiration—O_2-uptake was measured by means of Warburg's manometer. 0.2 ml. bacterial suspension, 0.3 ml. phosphate buffer, 0.3 ml. substrate and NaCl solution or distilled water to make a total volume of 3 ml. were added to the main chamber, and 0.1 ml. of 10 per cent NaOH was added to the central well. The gaseous phase was filled with air.

Na^+ Determination —Gravimetric method as sodium zinc uranyl acetate.

Cl^- Determination—Cl^- in solution was determined by silver nitrate and Cl^- in solid was estimated by the method of Koyama in which Cl was titrated by iodometry as Cl_2 after oxidation by $KMnO_4$ (*10*).

RESULTS

(*A*) *Estimation of "Intracellular Salt Concentration"*—The "Intracellular salt concentration" was estimated by the method indicated in Table I. The heavy cell suspension was stirred adequately with an

appropriate amount of water and centrifuged; Na^+ and Cl^- were determined with the supernatant. The molar concentration of Na^+ and Cl^- agreed well within experimental error. The residue of the

TABLE I

Treatment for Estimation of "Intracellular Salt Concentration"

Cell suspension
$+H_2O$
Centrifugation

Supernatant	Precipitate
(Determination of Na and Cl)	Evaporation at 100°
$Na_S = Cl_S$,	W_D
$NaCl_S$.	Dried material
	(Determination of Na and Cl)
	Na_P, Cl_P

centrifugation was evaporated at 100° and sodium and chlorine of the dried material were determined. In this case, contrary to the supernatant, significant difference between the molar concentrations of Na^+ and Cl^- was observed. This suggested the probable existence of a minute quantity of chlorine in the cell, which seemed to be reasonable because there could be only negligible amount of extracellular water in the wet precipitate of sample No. 5, Table II, if the cells themselves contained a considerable amount of chlorine. Thus the approximate "intracellular salt concentration" was determined in the following way:

$$\text{"intracellular salt concentration"} = \frac{\text{intracellular salt}}{\text{intracellular water}} = \frac{Na_{total} - Cl_{total}}{W_D - Cl_P/NaCl_S} \times 100$$

$Na_{total} = Na_S + Na_P$ $\quad Cl_{total} = Cl_S + Cl_P$

Na_S, Cl_S : mg. of NaCl corresponding to Na and Cl in the supernatant, respectively,

Na_P, Cl_P: mg. of NaCl corresponding to the total amount of Na and Cl in the precipitate, respectively,

$NaCl_S$: NaCl % in the supernatant,

W_D: mg. of water contained in the precipitate.

It must be mentioned that it is only for the sake of convenience that "intracellular salt concentration" was estimated as NaCl.

It may be concluded from Table II that:

(i) Intracellular salt concentration decreases with that in the medium; (ii) In our experimental conditions, the amount of intra-

TABLE II

Estimation of "Intracellular Salt Concentration"

	Number of sample		No. 1	No. 2	No. 3	No. 4	No. 5
	Added water	*mg.*	0	470	966	1,880	3,760
Supernatant	Na	*mg.*	129.1	141.8	146.8	154.5	161.9
Supernatant	NaCl ($NaCl_S$)	*per cent*	10.80	8.38	6.73	4.95	3.24
Precipitate	Na_P	*mg.*	49.8	37.0	31.2	23.7	16.7
Precipitate	Cl_P	*mg.*	34.4				2.2
Precipitate	Water W_D	*mg.*	491	464	479	468	473
Na_{total}		*mg.*	178.9	178.8	178.0	178.2	178.6
Cl_{total}		*mg.*	163.6	(····· 163.7 ·····)			164.0
Intracellular salt		*mg.*	15.4	15.1	14.3	14.4	14.6
Intracellular water		*mg.*	174	203	229	280	406
Intracellular salt concentration		*per cent*	8.9	7.5	6.3	5.2	3.6

Two g. of cell suspension were treated as indicated in Table 1 after addition of an appropriate amount of water. Amount of Cl in precipitate was determined only for sample No. 1 and No. 5; the average of No. 1 and No. 5 were given as the Cl_{total}.

cellular Na remained constant regardless of NaCl concentration in the medium; (iii) Water can freely diffuse from inside to outside the cells and *vice versa*.

It appears likely that the osmotic pressure is controlled in this way.

(*B*) *Substrate for the Respiration*—The O_2-uptake of the bacteria was measured with various substrates as shown in Table III. Generally speaking, amino acids provided good substrates and since peptone broth used as culture medium gave the best results, it was used as substrate for O_2-uptake in the following experiments.

(*C*) *Effects of NaCl on O_2-uptake*—As mentioned above, this organism can grow in considerably high salt concentrations, and we were interested in seeing the effect of the salt content of the medium on respiration. As

TABLE III

Respiration Substrates for Bact. pumilus var.

Substrate	O_2-uptake (*µl./30 minutes*)
None	31
Glucose	36
Formate	49
Glutamate	59
Leucine	59
Aspartate	56
Arginine	35
Casamic acid	125
Peptone broth	200

The final concentration of substrates was 0.01 *M*, excepting peptone broth which was 0.1%. pH 6.8. Temperature, 37°. NaCl concentration, 3%.

shown in Fig. 1, there was always a maximum of O_2-uptake between 5 and 7 per cent NaCl regardless of the nature of the substrates, *i.e.* no substrate, or formate or peptone broth substrate. On the contrary, glucose respiration of *E. coli*, a non-halotolerant organism, decreased suddenly as the salt content of the medium exceeded 2 per cent.

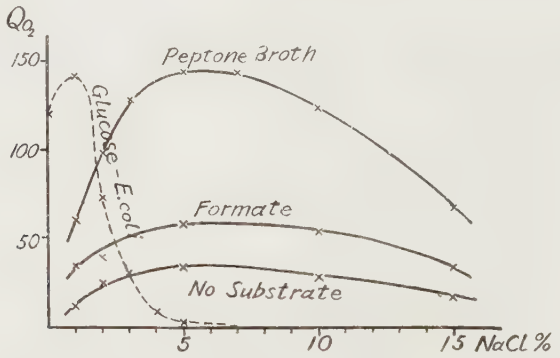

FIG. 1. Effects of NaCl on O_2-uptake.

O_2-uptake of *Bact. pumilus var.* and that of *E. coli* are represented by full lines and broken line respectively.

(*D*) *Effects of Salts on Some Enzymatic Activities*—It was observed that the salt concentration within cells reached a value of 8 per cent in

terms of NaCl when cultured in a medium containing 10 per cent NaCl and washed with 10 per cent saline, and that a maximum existed when the O_2-uptake for various substrates was plotted against the NaCl concentration of the medium. These facts suggested that the enzyme protein of this microrganism could be characterized from its behavior towards the salt concentration of the medium.

We therefore investigated the effect of the salt on some cell-free enzymes (Fig. 2). So far as our experiments were concerned, no enzyme for which an optimal salt concentration existed could be found. Robinson *et al* (*9*) have also indicated the difference between the optimal salt concentration for denitrification by living cells and by cell-free "nitritase". Though enzymes with a characteristic behavior could not be found in the present case, the existence of such an enzyme is not to be excluded.

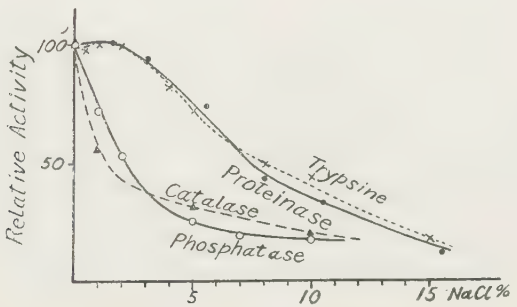

FIG. 2. Effects of salt on enzymatic activities.
Full line: Enzymes of *Bact. pumilus var.*
Dotted line: Trypsine as control.

(*E*) *Effects of Various Cations and Anions*—We next investigated whether the above mentioned effect of salt was restricted to NaCl. We chose K^+ and Mg^{++} in the forms of KCl and $MgCl_2$ as cations and NO_3^- and SO_4^{--} in the forms of $NaNO_3$ and Na_2SO_4 as anions and a maximum was found to exist likewise on the O_2-uptake—salt concentration curves. If each salt concentration was plotted against the isotonic NaCl concentration and not against ionic strength, it was found that the O_2-uptake curves coincided with each other, excepting the case of $MgCl_2$. In the latter case, the position of maximum was shifted towards lower concentrations; however, this is presumably due to Mg^{++} inhibition.

(*F*) *The Effects of Glucose*—The results from Expt. (*E*) sug-

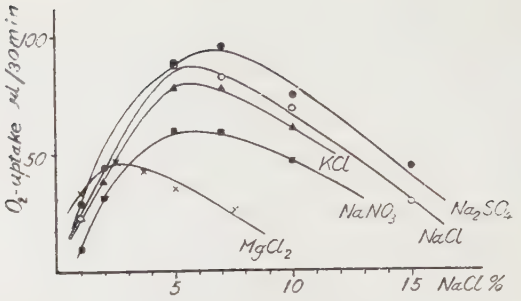

Fig. 3. Effects of various cations and anions. Abscissa is plotted against isotonic NaCl concentration.
—○—: NaCl, —▲—: KCl, —■— $NaNO_3$, —●— Na_2SO_4, —×—: $MgCl_2$.

gested the osmotic pressure to be the essential factor controlling the O_2-uptake; hence, the electrolyte was replaced by a non-electrolyte, glucose, (Fig. 4). In this case also we plotted the abscissa against isotonic NaCl concentration in place of glucose concentration. In the experiment with glucose only, the maximum of O_2-uptake was found at about 5 per cent as NaCl, though the activity was supressed generally. When we strengthened the osmotic pressure by adding glucose in 2 per cent NaCl, the maximum at about 5 per cent was clearly observed. This observation supported the view mentioned in the beginning of this section.

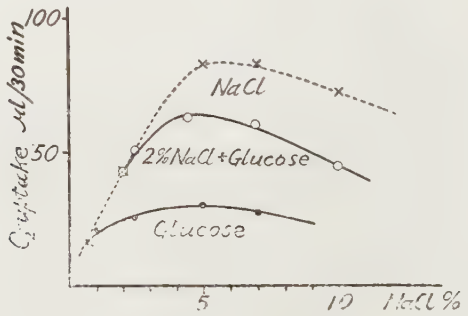

Fig. 4. The effects of glucose.
Abscissa is plotted against isotonic NaCl concentration.

(*G*) *The Influence of Preincubation and Ultrasonic Vibration on the Salt Effect*—As indicated in Fig. 5, we examined the salt effects on O_2-uptake respectively after the cell suspensions were preincubated in NaCl solution of various concentrations for 1 hour at 37° (Fig. 5).

While we could not find any difference between samples treated with 5 or 10 per cent saline and the untreated control, the maximum O_2-uptake was supressed remarkably by a cell preincubated in 1 per cent. When we incubated the cell suspension in various NaCl concentrations, a rapid decrease in vital counts was caused by less than 2 per cent (Table IV). Thus the remarkable change observed with the preincubated cells in 1 per cent can be accounted for by assuming that this treatment would be fatal for most of the bacteria and dead cells have only lower or rather low maximum of O_2-uptake.

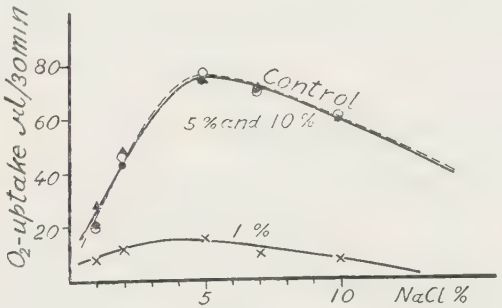

Fig. 5. The influence of preincubation on the salt effects.
— — ○ — —: control (untreated),
—●—, —▲—, —×—: preincubated in 10%, 5% and 1% NaCl solutions respectively.

Table IV

Vital Counts of Bacillus pumilus var.

NaCl concentration *per cent*	0.1	1	5	10	20
Vital count	1.3×10^5	5.4×10^5	1.2×10^7	2.0×10^7	2.0×10^7

Washing cells, originally 2.0×10^7, were incubated in saline with various NaCl concentration for 3 hours, and surviving colonies were counted in the conventional manner.

The following phenomenon supported this assumption: O_2-uptake of the cell suspension which was exposed to ultrasonic vibration decreased with increasing NaCl concentration of medium in a straightforward manner (Fig. 6).

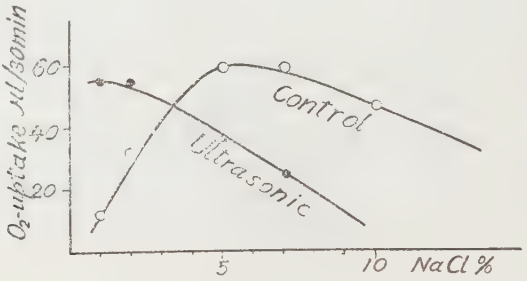

Fig. 6. The influence of ultrasonic vibration.

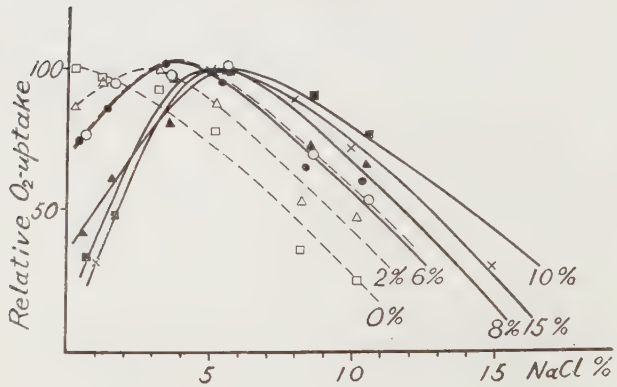

Fig. 7. Changes of salt effects related to culture condition.
Broken lines represent 20 hours cultures. —□—: 0%, —△—: 2%, —○—: 6%. Full lines represent 38 hours cultures, —●—: 6%, —▲—: 8%, —■—: 10%, —×—: 15%.

(*H*) *Changes of Salt Effects Related to Culture Conditions*—In preceeding sections, the test organism was cultured in the medium containing 10 per cent NaCl. As shown in Fig. 7, we investigated the O_2-uptake of suspensions of bacteria grown under varying salt concentrations and washed with saline having the same concentration as that of the medium respectively. In the case in which the bacteria were grown on a medium

containing no extra NaCl, the washing was carried out with a 1 per cent NaCl solution. The growth rate decreased as the salt concentration exceeded 8 per cent, and we harvested in cases of culture below 6 per cent after 20 hours and those above 6 per cent after 38 hours. We could not find significant differences between two samples of 6 per cent culture and we may neglect the effects of culture duration on O_2-uptake curve. Until the salt concentration in the culture medium reached 8 per cent, the optimum salt concentration for respiration was shifted towards a higher value, but no further increment was noted above 8 per cent.

We assumed these phenomena to be as follows; The variation of the " intracellular salt concentration " is caused by the salt concentration of culture medium. In fact, estimating sodium contents of cell pastes which grew in various salt concentrations, it is demonstrated that the salt content of cells and medium coincided at 1~2 per cent, while, as shown in Table II, in a 10 per cent culture the concentration of washing saline coincided with the intracellular concentration above 5 per cent.

DISCUSSION

When we began to investigate the halotolerancy of *Bacillus pumilus var.*, we at first faced a question as to whether this organism had an abnormally high salt concentration in the cells or not. Robinson *et al.* (9) reported that the salt concentration was considerably less than that of the medium in which they were growing. Our estimation of Na amount in the cells indicates that the intracellular Na concentration may reach a value of 8 per cent calculated in terms of NaCl, although it is usually less than that of the medium in which they are growing and varies with culturing conditions and the salt concentration of the suspension; on the other hand, Cl estimation gave a low value for intracellular Cl concentration. From these facts we consider that sodium presumably exists in high concentrations in the cells in forms other than NaCl. This point remains to be solved. The bacteria have a maximum for the O_2-uptake against the salt concentration of the medium, and these observations suggest the possibility that the enzyme protein of this organism could be characteristic in respect to its behaviour towards the ionic strength, *i.e.* optimum salt concentration for enzyme activity might appear at rather higher ionic strengths. Our results (catalase, phosphatase and proteinase) as well as that of Robinson's " nitritase ", were not consistent with this view. The appearance

of maximum in such biological activities of the living cells in respect to salt concentration is not found in most cell-free enzymes.

The interpretation proposed by Robinson that some intracellular energy mechanism maintains the difference between internal and external salt concentration in halophiles can not entirely be accepted in our case, since the oxygen uptake shows a maximum when there is little difference between the intracellular and surrounding Na concentrations.

Moreover, the following observations have been made:

1) In the resting cells the intracellular Na is rather in a " bound " state. Nevertheless, due to water diffusion into and out of the cells, the concentration varies with the change in salt concentration of the medium;

2) The maximum of O_2-uptake seems to be directed mainly by osmotic pressure, no matter whether an electrolyte or a nonelectrolyte is responsible for it;

3) The salt concentration of the culture medium affects the Na amount in the cells and the position of maximum O_2-uptake.

We assume that the water content in the cells, which is determined by the intracellular Na amount and NaCl concentration in the medium, affects the organization in the living cells and consequently the appearance of the O_2-uptake—salt concentration curve.

SUMMARY

1. We have estimated the amount of Na and Cl in the cells of an halotolerant bacterium, *Bacillus pumilus var.* The Na concentration depends on several factors and may reach a considerably higher value. It may be considered that there is little chlorine in the cells.

2. In resting cells, the intracellular Na amount is rather constant but when the NaCl concentration in the medium changes, the intracellular Na concentration varies with water diffusion into or out of the cells.

3. The O_2-uptake of this microrganism has a maximum in respect to the NaCl concentration of the medium.

4. We have observed the effects of various salts and glucose on the maximum. The results indicated that the main factor which determined the position of the maximum is probably the osmotic pressure.

5. No optimal salt concentration existed for enzymatic activities of the bacteria, catalase, phosphatase and proteinase.

We are greatly indebted to Mr. H. Yamada for a gift of the bacterial strain, to Prof. S. Hosoya and Dr. M. Soeda for its identification and to Prof. F. Egami for his kind advices and encouragements.

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THE MECHANISM OF ACONITASE ACTION

I. THE STEADY-STATE ANALYSIS AND THE KINETIC THEORY*

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(Received for publication, April 13, 1953)

Aconitase is one of the most important enzymes but its reaction mehcanism is not clear, and it is reported as a single enzyme or sometimes a multi-enzyme composed of two enzymes. But the author could not find any exact evidence based on the theoretical ground because of the general lack of the synthetic examination and the disregard of the limits of the accuracy of the methods of estimations. The author investigated more carefully that it is a single enzyme, the details of which will be reported.

EXPERIMENTAL

Rabbit Liver Aconitase—A 20 g. portion of frozen rabbit liver was homogenized with 60 ml. of 0.01 *M* phosphate buffer of pH 7.4. To the homogenate 60 ml. of saturated ammonium sulfate solution was added, and the mixtur was centrifuged at 4,000 r.p.m. for 20 minutes. The enzyme was precipitated from the supernatant solution by adding solid ammonium sulfate to 80 per cent saturation. The precipitate was dissolved in 10 ml. of the same buffer, then heated quickly to 50–51° and maintained for 10 minutes at this temperature. After cooled the innert precipitate was centrifuged off. To the supernatant 3 ml. of suspension of alumina gel Cγ (2 per cent dry weight) was added, then alumina was centrifuged off. The supernatant was dialyzed to distilled water for 2 hours and used as the enzyme solution throughout this experiments. The preparation was not so highly active, but it was stable and reproducible and could be stored in frozen state without considerable loss of activity.

Isocitric dehydrogenase was prepared from pig heart mainly according to Ochoa (*1*).

Chemical Preparations—TPN was prepared from pig livers by the method of Le Page *et al.* (*2*). *dl*-Isocitric acid lactone (m.p. 162°) was prepared by the method of Pucher (*3*), and the concentration of its acid salt is represented as the concentration of *d*-isomer. *Cis*-aconitic acid m.p. 128° was prepared according to Krebs *et al.* (*4*).

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* This work was presented at the 5th Meeting of Symposia on Enzyme Chemistry, held at Osaka in July, 1952, and printed in Japanese.

Other materials were reagent grade commertial preparations.

Methods—Experiments were performed at 25° unless otherwise stated. Usualy the enzyme action was started by adding the substrate solution to the same volume of the enzyme solution in 0.02 *M* phosphate buffer at pH 7.4 after temperature equilibrium.

(a) Reactions determined by citrate estimation: The total volume of 5–15 ml. of the reaction mixtures was used, the reaction was stopped by adding 1 ml. of 20 per cent trichloroacetic acid, and citrate was estimated by the method of Natelson *et al.* (*5*).

(b) Reactions determined by *cis*-aconitate estimation: The total volume of 2.5–3 ml. of the reaction mixture was used. The reactions were performed mainly in quartz cells of 1 cm. light path according to the method of Racker (*6*), but for the estimation of the activation energy, the reactions were performed without the optical system, and, after the reactions were stopped by adding 1 *N* H_2SO_4 to the reaction mixtures, the absorption was measured.

(c) Reactions determined by isocitrate estimation: The same mixtures as (a) were used. The reactions were stopped by adding 0.8 ml. of 1 *N* NaOH and 0.2 ml. of 0.1 *M* KCN to the reaction mixtures. After a few hours the mixtures were neutralized and the isocitrate in the centrifugal supernatant was estimated by the method of Ochoa (*1*).

RESULTS

Initial Reactions—When the quantity of the product is far less than the Michaelis constants of the three substrates and the inhibition by the product is negligible, the reaction is called the initial reaction. All the experiments, except in case two kinds of the substrates were mixed, were the reactions under this condition. In Figs. 1 and 2, the amounts of the reaction products show the first order relation against time when they do not go beyond the Michaelis constants of the substrates. And the ratio of the rate producing *cis*-aconitate from isocitrate to that producing citrate from isocitrate is about 2.5 (from 2.3 to 2.7), and the ratio of the rate producing isocitrate from *cis*-aconitate to that producing citrate from *cis*-aconitate is about 1.2 (from 1.1 to 1.3).

Michaelis Constants (*K*)—The Michaelis constants of the three substrates were obtained from the following five reactions at 25° and at pH 7.4 by the method of Leinweaver *et al.* (*7*). The constant of citrate, K_C, was 9×10^{-4} *mole/lit.*, estimated from the reaction producing *cis*-aconitate from citrate. That of *cis*-aconitate, K_A, was 9×10^{-5} and 1×10^{-4} *mole/lit.*, estimated from the reaction producing citrate from *cis*-aconitate and that of *cis*-aconitate consumption, respectively. In the following studies the former value will be used, as the accuracy

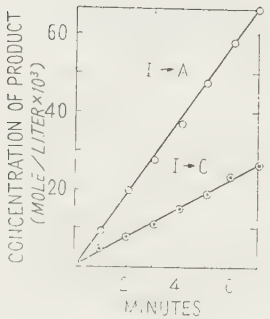


FIG. 1. Initial reactions starting from isocitrate.

-⊙-⊙- Citrate formation: 2.5 ml. 0.04 *M* isocitrate +2.5 ml. enzyme solution.

-O-O- *cis*-Aconitate formation: The total 3 ml. the same reagent concentrations as above.

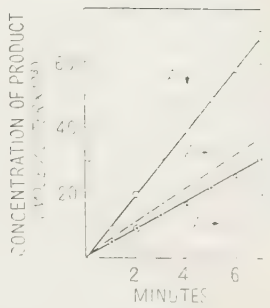


FIG. 2. Initial reaction starting from *cis*-aconitate.

-⊙-⊙- Citrate formation: 2.5ml. 0.008 *M cis*-aconitate+ 2.5ml. enzyme solution.

-O-O- *cis*-Aconitate consumption: The total 3 ml. the same reagent concentrations as above.

-•- Isocitrate formation: Calculated from *cis*-aconitate consumption and citrate formation.

of the later value is somewhat low. The constant of isocitrate, K_I, was 3.2×10^{-4}*mole/lit.*, estimated from the reaction producing citrate from isocitrate and that producing *cis*-aconitate from isocitrate. In this case both methods gave the same value.

The Activation Energies—The experiments were made in the presence of enough substrate, and they satisfied the Arrhenius' equation throughly between the temperature of 5–30° and at pH 7.4. The value of the reaction producing *cis*-aconitate from citrate and that of the reaction producing citrate from *cis*-aconitate were 13 and 12 *kcal./mole.*, respectively and those of the reactions producing citrate from isocitrate and *cis*-aconitate from isocitrate were the same, and they were 12 *kcal./mole.*, each.

The Rate of the Formation of the Third Substrate in Case Two Substrates were Mixed in Several Defined Ratios:

(*a*) *In the Presence of the Sufficient Amount of the Substrates*—In order to solve the problem whether it is a single enzyme or not, the measuerment of the rate of the reaction when the substrates are mixed, gives an important knowledge. Fig. 3. shows the rate of *cis*-aconitate formation from the mixtures of citrate and isocitrate. The circles on full

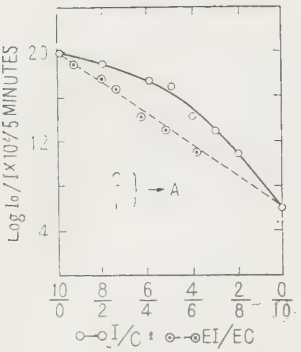


FIG. 3. *cis*-Aconitate formation from the mixtures of citate and isocitrate.

1.5 ml. substrates mixed in various ratio+1.5 ml. enzyme solution. The increase of absorption was measured for 5 min. Fraction in abscissa indicates the ratio of isocitrate to citrate or of EI to EC. The actual meanings of numerators and dominaters are respectiuely the concentrations of isocitrate and citrate taking 0.003 *M*. for one unit. For example 8/2 means the mixture of 0.024 *M* isocitrate and 0.006 *M* citrate at final concentration.

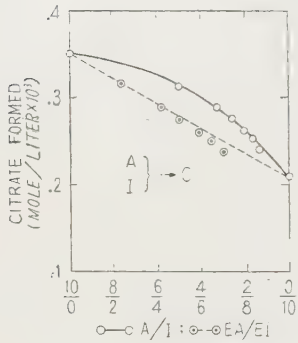


FIG. 4. Citrate formation from the mixtures of *cis*-aconitate and isocitrate.

2.5 ml. substrate mixed in various proportions +2.5 ml. enzyme sulution. Other meanings are similar to Fig. 3, but *cis*-aconitate and isocitrate were added and citrate was estimated after 10 min.

line show the reate of *cis*-aconitate formation when isocitrate and citrate are mixed in the corresponding ratios in abscissa. When the abscissa is scaled by the product of the ratio of the amount of the two substrates and the reciprocal of the ratio of Michaelis constants of the two substrates, circles move horizontaly to dotted circles, and in this case the ratio in the abscissa shows the ratio of the two enzyme-substrate complexes as proved later (Eq. 4).

The similar experiments were performed concerning the reaction producing citrate when *cis*-aconitate and isocitrate were mixed and the reaction producing isociatrate when citrate and *cis*-aconitate were mixed. The result of the former experiment is shown in Fig. 4.

These experimental results show that the rate of the formation of a product from mixed substrates lies between the larger rate and the smaller rate obtained in the case of a single substrate, and that the value indicated by the dotted circles lies on a straight line if the abscissa

is scaled by the ratio of the concentrations of the two enzyme-substrate-complexes.

(*b*) *In the Presence of the Small Amount of Substrates*—Two substrates were mixed in the ratio of their respective Michaelis constants (Eq. 6), and their concentrations were defined by n which shows the ratio of the Michaelis constant to the concentration of substrate (Eq. 10). Concerning the rate of the formation of *cis*-aconitate from the mixtures of citrate and isocitrate, the results shown in Table I and Fig. 5 were obtained.

TABLE I

Relations between the Rate of cis-Aconitate Formation and Concentrations of Substrate Mixtures

Single substrate	Concentration (*mole per lit.*)	v	*Vmax.*	V_C+V_I
Citrate	9×10^{-3}	17	19	80
Isocitrate	3.2×10^{-3}	56	61	

Mixed substrates No.	n	v	$\frac{V_C+V_I}{v}$	
			Observed	Calculated
1	0.1	38	2.1	2.1
2	0.2	35	2.3	2.2
3	0.5	31	2.6	2.5
4	1.0	26	3.1	3.0
5	2.0	21	4.0	4.0

1.5 ml. substrate or substrates and 1.5 ml. enzyme solution. Rate v as $1000\times\log I_0/I$ per min. at 240 mμ. These values are means of 5 determinations in 1 min. interval except in No. 5, in which the value is twice the mean of 4 determinations in 30 sec. interval. *Vmax* are calculated from v and K.

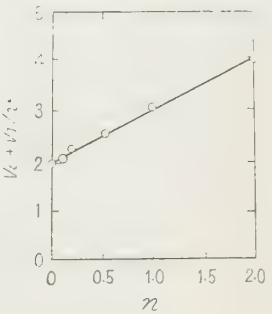


FIG. 5. The relation between the rate of formation of *cis*-aconitate and the concentration of substrate mixtures.

Meanings of this figure are read in the text and Table I.

DISCUSSION

Concerning the mechanism of aconitase, there are the following

possibilities: (a) Aconitase is a single enzyme; there are two possible mechanims, in the first case only one activated complex is formed and in the second case different activated complexes are formed when the products are different; (b) Aconitase is composed of two members; and in this case there are three possibilities according to different processes of reactions; (c) Aconitase is composed of three members.

Now the author supports the view that the first mechanism takes place from the following reasons:

(a) All the initial reactions did not show a lag period. Though similar studies were already done by Martius *et al.* (*8, 9*), the discussions without consideration of the Michaelis constant of *cis*-aconitate and the error of the optical rotation method are meaningless. The result reported by Krebs *et al.* (*10*) does not disprove the first mechanism as will be stated in the following paper, but this reason does not deny the possibility of the last mechanism;

(b) The Michaelis constants of all the reactions can be determined by the method of Leinweaver *et al.* (*7*). This is not possible when the enzyme is composed of two members. And the findings, that the values were specific to the starting substrate and were independent from the reactions by which they were measured, is one of the important evidences of the first mechanism;

(c) The fact, that the activation energies are independent from the reaction by which they are measured even if products are different, can easily be understood when the enzyme is supposed to be one and the activated complex also only one even when different products are formed;

(d) The results presented in Table I., Figs. 3, 4, and 5. can not be explained by the mechanisms of two or three enzymes but can easily be explained as follows by the first mechanism. Some relations between the rate constants are discussed as follows.

The reactions producing *cis*-aconitate from citrate and isociatrate are composed of the following two sequences.

$$\left.\begin{array}{l} \mathrm{E+C} \underset{k_{2C}}{\overset{k_{1C}}{\rightleftarrows}} \mathrm{EC} \xrightarrow{k_{3C}} \mathrm{E+C+A+I} \\ \mathrm{E+I} \underset{k_{2I}}{\overset{k_{I}}{\rightleftarrows}} \mathrm{EI} \xrightarrow{k_{3I}} \mathrm{E+C+A+I} \end{array}\right\} \quad \text{Eqs. 1.}$$

(E: free enzyme; C, A, I: substrates; EC, EI: enzyme-substrate-complexes; k_1, k_2, k_3: rate constants, suffixed by the starting substrate;

ε: total enzyme.)

It is nothing but considering the mode of reaction shown later that substrates starting are added to substrates formed. The k_3s are actualy the rate of activation of three enzyme-substrate-complexes. All, in the bracket, show concentrations. As proved later, the relation $k_1 \gg k_2 \gg k_3$ consists, thus Eqs. 2 can be led in the stationary state.

$$\left.\begin{aligned}(\mathrm{EC}) &= \frac{[(\varepsilon)-(\mathrm{EC})-(\mathrm{EA})-(\mathrm{EI})]\ (\mathrm{C})}{K_\mathrm{C}} \\ (\mathrm{EI}) &= \frac{[(\varepsilon)-(\mathrm{EC})-(\mathrm{EA})-(\mathrm{EI})]\ (\mathrm{I})}{K_\mathrm{I}}\end{aligned}\right\} \quad \text{Eqs. 2.}$$

To solve Eqs. 2 concerning (EC) and (EI),

$$\left.\begin{aligned}(\mathrm{EC}) &= \frac{[(\varepsilon)-(\mathrm{EA})]\ (\mathrm{C})\ K_\mathrm{I}}{K_\mathrm{C}K_\mathrm{I}+K_\mathrm{C}(\mathrm{I})+K_\mathrm{I}(\mathrm{C})} \\ (\mathrm{EI}) &= \frac{[(\varepsilon)-(\mathrm{EA})]\ (\mathrm{I})\ K_\mathrm{C}}{K_\mathrm{C}K_\mathrm{I}+K_\mathrm{I}(\mathrm{C})+K_\mathrm{C}}\end{aligned}\right\} \quad \text{Eqs. 3.}$$

Then $$\frac{(\mathrm{EC})}{(\mathrm{EI})} = \frac{(\mathrm{C})}{(\mathrm{I})} \cdot \frac{K_\mathrm{I}}{K_\mathrm{C}} \qquad \text{Eq. 4.}$$

On the other hand the rate of formation of *cis*-aconitate when substrates are mixed is as follows.

$$v = k_{3\mathrm{C}}(\mathrm{EC}) + K_{3\mathrm{I}}(\mathrm{EI}) \qquad \text{Eq. 5.}$$

Dotted line in Fig. 3 is the theoretical line obtained from Eqs. 4 and 5, but concerning k_3 and K the determined values of the rate of formation of *cis*-caconitate and that of Michaelis constants are used respectively. The determined values in various conditions rest on the theoretical line. In Fig. 4 the situations are analogous. Further experiment shown in Fig. 5 and Table I was performed in the following special conditions. When two substrates are mixed in the next proportion, Eqs. 7 can be led.

$$\frac{(\mathrm{I})}{(\mathrm{C})} = \frac{K_\mathrm{I}}{K_\mathrm{C}} \qquad \text{Eq. 6.}$$

$$\left.\begin{aligned}(\mathrm{EC}) &= \frac{[(\varepsilon)-(\mathrm{EA})]\ (\mathrm{C})}{K_\mathrm{C}+2\ (\mathrm{C})} \\ (\mathrm{EI}) &= \frac{[(\varepsilon)-(\mathrm{EA})]\ (\mathrm{I})}{K_\mathrm{I}+2\ (\mathrm{I})}\end{aligned}\right\} \quad \text{Eqs. 7.}$$

In the earlier period of reactions as $(\mathrm{EA}) \ll (\varepsilon)$,

$$(\mathrm{EC}) = \frac{(\varepsilon)\ (\mathrm{C})}{K_\mathrm{C}+2\ (\mathrm{C})}; \quad (\mathrm{EI}) = \frac{(\varepsilon)\ (\mathrm{I})}{K_\mathrm{I}+2\ (\mathrm{I})} \qquad \text{Eqs. 8.}$$

From Eqs. 5 and 8

$$v=\frac{k_{3C}\ (\varepsilon)\ (C)}{K_C+2\ (C)}+\frac{k_{3I}\ (\varepsilon)\ (I)}{K_I+2\ (I)} \qquad \text{Eq. 9,}$$

in which if (C) is difined by K_C/n,

$$n=\frac{K_C}{(C)}=\frac{K_I}{(I)} \qquad \text{Eq. 10.}$$

From Eqs. 9 and 10,

$$v=\frac{k_{3C}\ (\varepsilon)+k_{3I}\ (\varepsilon)}{n+2} \qquad \text{Eq. 11.}$$

On the other hand, the maximum rate *Vmax.*, when the substrate is not mixed, is shown as V_C and V_I respectively to the reaction strating from citrate and isocitrate, then,

$$V_C=k_{3C}(\varepsilon);\quad V_I=k_{3I}(\varepsilon) \qquad \text{Eqs. 12.}$$

From Eqs. 11 and 12,

$$\frac{V_C+V_I}{v}=n+2 \qquad \text{Eq. 13.}$$

Eq. 13 shows the relation between the concentration of substrates and the rate of reaction when the relations presented by Eqs. 6 and 10 exist. Fig. 5 and Table I show that this relation is satisfied. For explaining the results in Figs. 3 and 4 the absolute value is out of question, if only the ratio of the determined values of Michaelis constants coinsides with that of K_S in equations, but the second fact shows that they should coincide ever on the absolute values.

Next, the relation between rate constants are discussed. As Michaelis constant represented by Eq. 14 is considerably small, Eqs. 15 can be obtained.

$$K=k_2/k_1+k_3/k_1 \qquad \text{Eq. 14.}$$

$$k_1 \gg k_2;\quad k_1 \gg k_3 \qquad \text{Eqs. 15.}$$

In order to clear the relation between k_2 and k_3, the reactions producing *cis*-aconitate either from citrate or isocitrate are taken into consideration as an example. As the process producing *cis*-aconitate by the decomposition of the activated complex is common, the fact, that the rate producing *cis*-aconitate from citrate and isocitrate is different even if there are enough substrate, proves that the rate of activation of the enzyme-substrate-complex determines the rate of *cis*-aconitate formation. In the case starting from citrate this rate is k_{3C} (EC). Then the rate of *cis*-aconitate formation from enzyme-*cis*-aconitate-

complex, k_{2A} (EA)—k_{1A} (EC), is greater than k_{3C} (EC), and thus

$$k_{2A}(EA) \gg k_{3C}(EC) \qquad \text{Eq. 16.}$$

In the earlier period of reactions, (EA)$\ll$(EC), then

$$k_{2A} \gg k_{3C} \qquad \text{Eq. 17.}$$

On the other hand, k_{3C}: k_{3I} is the ratio of the initial rate producing *cis*-aconitate from citrate to that from isociatrate by the same enzyme preparation, and it is shown to be 1:3.3 from Fig. 3.

Equally, k_{3A}: $k_{3I}=5 : 3.3$ from Fig. 4, then

$$k_{3C}: k_{3A}: k_{3I}=1 : 5 : 3.3 \qquad \text{Eq. 19.}$$

Then $k_{2A} \gg k_{3A}$ Eq. 20.

In general $k_2 \gg k_3$ Eq. 21.

Then $k_1 \gg k_2 \gg k_3$ Eqs. 22.

Also the next relation can easily be led, in which k_4 is the rate of decomposition of the activated-complex.

$$k_3 \ll k_4 \qquad \text{Eq. 23.}$$

From these results and some other evidences reported in the following papers, the mechanims of aconitase action is considered as follows:

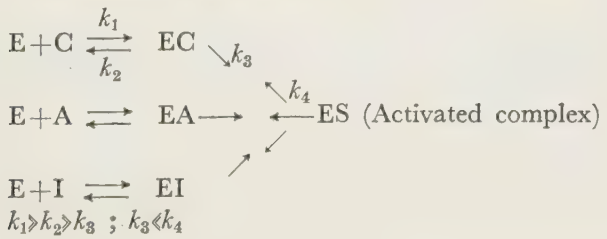
(a) Only one enzyme is involved and the three substrates combine competitively and reversibly at the same reactive center of the enzyme molecule, and form enzyme-substrate-complexes;

(b) Three kinds of enzyme-substrate-complexes are activated to one activated complex. The rate of activation is fixed on each enzyme-substrate-complex and independent mutualy. They determine the rates of reactions under the presence of enough substrate;

(c) The activated complex produces three kinds of enzyme-substrate complexes, the ratio of which is constant under the definite condition. But the ratio is statistic;

(d) The enzyme and the substrates combine specifically and three dimentionally.

To diagramatize this theory:



SUMMARY

Aconitase action was synthetically examined using the rabbit liver enzyme preparation and the following conclusions were obtained.

1. In the initial stage, all the reactions did not show any lag period.

2. Michaelis constants of three substrates were measured at 25°, pH 7.4. They were 9×10^{-4}, 9×10^{-5} and 3.2×10^{-4} *mole/lit.* respectively for citrate, *cis*-aconitate, and isocitrate. They were specific to the substrates starting regardless of the reaction by which they were measured.

3. Activation energies were determined. They were 13, 12, and 12 *kcal./mole* for the reaction starting from citrate, *cis*-aconitate and isocitrate, respectively. They were also specific to the substrate starting and independent from the reaction by which they were measured.

4. When two substrates were mixed, the rate of formation of the third substrate lay between the larger rate and the smaller rate obtained in the case of a single substrate. These and other results indicate that only one enzyme is involved and the three substrates combine competitively and reversively at the same reactive center of one enzyme.

5. One enzyme and one activated complex theory of aconitatse action is proposed.

The author should like to thank Dr. H. Fukumi, Dr. D. Mizuno, and Dr. H. Uchida.

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THE MECHANISM OF ACONITASE ACTION

II. SOME EVIDENCES SUPPORTING ONE ENZYME AND ONE ACTIVATED COMPLEX THEORY AND THE COMPARATIVE STUDIES ON FUMARASE ACTION.*

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In the previous report (*1*), the author concluded that aconitase is a single enzyme and proposed the following kinetic mechanism.

$$\begin{array}{l} E+C \underset{k_2}{\overset{k_1}{\rightleftarrows}} EC \quad \searrow k_3 \\ \qquad\qquad\qquad\quad \nwarrow k_4 \\ E+A \rightleftarrows EA \longrightarrow \longleftarrow ES \\ E+I \rightleftarrows EI \qquad \nearrow \swarrow \end{array}$$

$k_1 \gg k_2 \gg k_3$; $k_3 \ll k_4$.

(Abbriviations are the same as those in the previous report.)

In the present communication some experimental facts will be added which are regarded to support the above kinetic mechanism, and the comparative study of the mechanism of aconitase and fumarase will be reported.

EXPERIMENTAL

Following materials and methods are added to those described in the previous report (*1*).

Pig heart aconitase—The enzyme preparations were obtained from frozed pig heart according to the method of Buchanan *et al.* (*2*). The purification was stopped at the stage of the second ethanol precipitation. Some preparations were preserved at -2° for 20 days and this aged preparation sometimes showed some different nature from the fresh one. Bacterial aconitase—Cell free aconitase was prepared from *Proteus vulgaris* and *Brucella bronchseptica* by the glass powder grinding method. Yeast aconitase—The enzyme preparations were obtained from dried baker's yeast according

* This work was presented at 5th Meeting of Symposia on Enzyme Chemistry, in Osaka, July, 1952, and printed in Japanese.

to the method of Racker (*3*), but they could not be divided into two fractions which showed different ratios of the rate of formation of *cis*-aconitate from citrate and isocitrate.

Fumarase preparations—All aconitase preparations used in this study had more or less of fumarase activity, and these preparations were used to observe the action of fumarase.

Method—Isocitric acid was determined by the optical rotation method according to Eggleston *et al.* (*4*). Fumarase action was determined by the measurment of absorption of ultra violet ray accoding to Racker (*3*), but sometimes fumarate was measured by the absorption at 285 mμ in order to increase the amount of fumarate added as much as possible. For the same reason, in some experiments *cis*-aconitate was estimated by the absorption at 250 mμ.

RESULTS

Throughout the Sections from I to IV, only the rabbit liver enzyme preparations were used at 25°, pH 7.4.

I. The Maximum of cis-Aconitate Produced and the Maximum Rate of Formation of Citrate in the Case Starting from Isocitrate—When the reaction started from isocitrate, isocitrate decreased, citrate continued to increase, and *cis*-aconitate showed a muximum at a certain point of time as illustrated in Fig. 1. When *cis*-aconitate reached the maximum, the ratio of the concentration of the three substrates estimated was as follows.

$$(C) : (A) : (I) = 17 : 17 : 66.$$

And the rate of formation of citrate was not maximum at the begining of the reaction but showed maximum when the amount of *cis*-aconitate reached the maximum.

II. The Maximum of Isocitrate Produced in the Case Starting from cis-Aconitate—The solution of rabbit liver enzyme in 0.02 *mole/lit.* phosphate buffer of pH 7.4 was mixed with the same amount of 0.01 *mole/lit. cis*-aconitate solution and the concentrations of the substrates were measured with certain intervals. In this case isocitrate was measured by the optical rotation method. *cis*-Aconitate decreased, citrate continued to increase, and isocitrate showed a muximum at a certain point of time. When *cis*-aconitate reached the maximum, the ratio of the concentration of the three substrates estimated was a follows.

$$(C) : (A) : (I) = 45 : 20 : 35.$$

The main feature was almost the same as the results of the pig liver preparation by Martius (*5*).

III. The Ratio of cis-Aconitate to Isocitrate in Case cis-Aconitate and

Isocitrate are Mixed in Various Ratios and cis-Aconitate neither Increases nor Decreases—In order to obtain this ratio, the experiment was performed as shown in Fig. 2. The abscissa shows the ratio of isocitrate to *cis*-aconitate and scaled as indicated and the ordinate shows the rate of increase or decrease of *cis*-aconitate. The following value was obtained from the intersecting point of the determined curve and the horizontal line.

$$(A) : (I) = 1 : 3.7$$

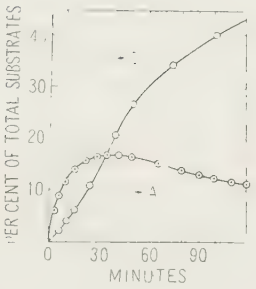

FIG. 1. The course of formation of citrate and *cis*-aconitate from isocitrate.
-○-○- Citrate formation: 2.5 ml. 0.004 *mole/lit.* isocitrate +2.5 ml. enzyme solution.
-◉-◉- *cis*-Aconitate formation: Total 3 ml. the same reagent concentrations.

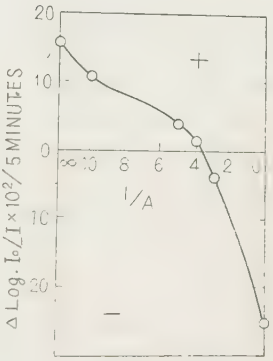

FIG. 2. The formation and the consumption of *cis*-aconitate when *cis*-aconitate and isocitrate are mixed in various ratios.
1 ml. enzym solution +1 ml. 0.0009 *mole/lit.* *cis*-aconitate+1 ml. various concentrations of isocitrate, its concentrations are defined by the ratio to *cis*-aconitate. Absorption was measured at 250 mμ.

IV. The Ratio of Citrate to cis-Aconitate in Case Citrate and cis-Aconitate are Mixed in Various Ratios and cis-Aconitate neither Increases nor Decreases—Fig. 3 shows the results of the similar experiment to Section III but it is performed with the mixture of citrate and *cis*-aconitate. The following value was obtained.

$$(C) : (A) = 45 : 1$$

V. Some Properties of the Enzyme Preparations Other than That of Rabbit

Liver—The rate of formation of the third substrate was measured when two substrates were mixed. The results are summarized in Fig. 4. The meanings and the experimental conditions are the same as Fig. 3 of Report I, but the ordinate shows the relative rate of *cis*-aconitate formation in varios conditions, the rate of *cis*-aconitate formation from citrate being taken as one unit. Curves A, B and C show the results obtained respectively by the enzyme preparations of *Proteus vulgaris*, baker's yeast and *Brucella bronchseptica*. Curves D and E show respectively those of the fresh and aged preparations of pig heart. Curve E type was not always obtained when these preparations were preserved in the same condition. The rate starting from the single substrate was always the largest or the smallest.

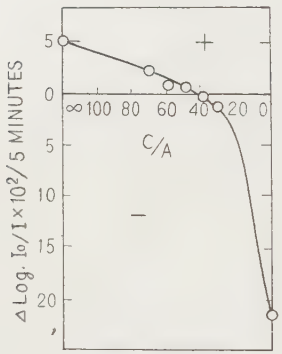

FIG. 3. The formation and the consumption of *cis*-aconitate when *cis*-aconitate and citrate are mixed in various ratios.

All conditions were the same as Fig. 2, but citrate was added instead of isocitrate.

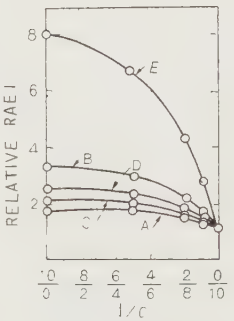

FIG. 4. Relative rate of *cis*-aconitate formation from the mixtures of citrate and isocitrate in various ratios.

The ordinate shows the relative rate, the abscissa shows the ratio of isocitrate to citrate. Curve A: *Proteus* enzyme. B: Yeast enzyme. C: *Brucella* enzyme. D: Fresh pig heart enzyme. E: Aged pig heart enzyme. The conditions were the same as Fig. 3 of Report I (*1*).

VI. The Michaelis Constants of Fumarase—Two Michaelis constants of the fresh preparation of pig heart fumarase were detertermined by the method of Leinweaver *et al.* (*6*), those of malate and

fumarate were respectively 3×10^{-3} and 2×10^{-3} *mole/lit.* at 20°, pH 7.4. The former value was obtained from the rate of formation of fumarate measured at 240 mμ, and the latter was obtained from the rate of decrease of fumarate measured at 285 mμ.

VII. The Ratio of the Rate Producing Malate from Fumarate to That Producing Fumarate from Malate in the Presence of the Same Amount of Enzyme—1.5 ml. of the enzyme solution were mixed with 1.5 ml. of 0.02 *mole/lit.* fumarate and 0.1 *mole/lit.* malate, respectively. The rate of change of the absorption of ultra violet ray at 285 mμ was measured at 20°, pH 7.4, and the rate of decrease of absorption of the former mixture was compared with the rate of increase of that of the latter. The ratios of these rates were considerably different when various preparations were used, and the values 3, 4.8, 3.4 and 4.3 were obtained respectively by the fresh and aged preparations of pig heart, the rabbit liver preparation and the *Proteus vulgaris* preparation.

DISCUSSION

The Theoretical Considerations of the Method to Explain the Experimental Results—If the author's theory of the reaction mechanism is correct, all the experimental facts must be explained using the determined values of the rate constants obtained in the preceeding paper (*1*). At first, the general method by which the experimental results are explained will be described.

In the first place, the ratio of the reciprocals of the three Michaelis constants is

$$1/K_C : 1/K_A : 1/K_I = 1 : 9 : 3.$$

Then the ratio of the concentrations of the three enzyme-substrate complexes is

$$(EC) : (EA) : (EI) = 1(C) : 9(A) : 3(I)$$

Whereas there is the following relation between the rates of activation of enzyme-substrate complexes (Eq. 19 in Report I (*1*)).

$$k_{3C} : k_{3A} : k_{3I} = 1 : 5 : 3.3$$

Then the ratio of the rates, when the three substrates are activated, is

$$1\ (C) : 9\times5\ (A) : 3\times3.3\ (I)$$

so $$1\ (C) : 45\ (A) : 10\ (I) \qquad \text{(Relation A)}$$

On the other hand, the ratio of the rates of formation of enzyme-substrate complexes by decomposition of the activated complex is ob-

tained from the ratio among the rate of formation of two substrates from a single substrate. From Figs. 1 and 2 of Report I, the following values are obtained.

$$k_{4C} : k_{4A} = 1 : 2.5 \text{ and } k_{4C} : k_{4I} = 1 : 1.2,$$

then $k_{4C} : k_{4A} : k_{4I} = 1 : 2.5 : 1.2,$

shown by percentage 22 : 52 : 26. (Relation B)

Thus, when a certain substrate or substrates neither increase nor decrease, the percentage of the above mentioned substrate or the substrates obtained from Relation A must coincide with that of Relation B, regardless of the increase or the decrease of the other substrates.

The Explanation of the Eexperimental Results—As an example, the experiment described in Fig. 1 is taken up. At the maximum of *cis*-aconitate, Relation A, shown in percentage, becomes 1 : **53** : 46. The quite agreeable coincidence is seen with the value of Relation B concerning *cis*-aconitate. This shows that there is neither momentary increase nor decrease of *cis*-aconitate at this point. And values of citrate and isocitrate show that citrate is going on increase and isocitrate is going on decrease.

The explanations of experimental results by such method are summarized in Table I. The figures shown by gothic style coincide with the respective value in Relation B.

TABLE I

Explanations of Experimental Facts

	C : A : I	Relation A
Maximum of *cis*-aconitate in Sec. I.*	17 : 17 : 66	1 : **53** : 46
Maximum of isocitrate in Sec. II.*	45 : 20 : 35	3 : 70 : **27**
Equilibrium of *cis*-aconitate in Sec. III.*	0 : 1 : 3.7	0 : **55** : 45
in Sec. IV.*	45 : 1 : 0	50 : **50** : 0
Equilibrium point by Krebs (*7*)	89.5 : 4.3 : 6.2	**25** : **55** : **20**
by Martius (*5*)	89.2 : 3.1 : 7.7	**29** : **46** : **25**

*the section number in ⟪ RESULTS ⟫.

Furthermore the maximum of the the rate of citrate formation shown in Fig. 1 is easily explained by the increase of the rate of acti-

vation of the enzyme-substrate complexes resulted from the increased amount of *cis*-aconitate. The experimental results of Krebs *et al.* (*8*) by which they denied one enzyme mechanism can be explained clearly in the similar way.

Differences among the Preparations—The relations among various rate constants obtained from different enzyme preparations are considerably different quantitatively, but the results in Fig 4 prove aconitase is a single enzyme. Concerning the difference of enzyme preparations from the same original material, Racker (*3*) pointed out the presence of two types of enzymes, but Fig. 4 proves that the difference is not derived from the change of the relative concentrations of two or three different enzymes also in this case. Such a great change as in the case of the pig heart preparations was not observed in the case of rabbit liver preparations. The difference between enzyme preparations should be noted but it is just the same as in the case of fumarase which is going to be compared.

Comparative Studies on Fumarase Action—Concerning fumarase, the similar mechanism as aconitase is presented.

$$\begin{array}{ccccc} & & & \searrow^{k_3} & \\ E+F & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & EF & & ES \\ & & & \nearrow_{k_4} & \\ E+M & \rightleftharpoons & EM & & \end{array}$$

(Abbriviations and meanings are similar to those used in the case of aconitase)

Like aconitase $k_1 \gg k_2$ and $k_1 \gg k_3$ can be obtained, but the relation between k_1, k_2 and k_3 and that of k_3 and k_4 can not be obtained. Now the relation $k_1 \gg k_2 \gg k_3$ and $k_3 \ll k_4$ are supposed as similar to aconitase. Then k_3 becomes rate determining factor if the quantity of the substrate is enough. As mentioned before, the ratio of the rate producing malate fumarate to that producing fumarate from malate was 3 with the fresh pig heart preparations. This value is the ratio of the rate producing malate from the enzyme fumarate complex to that producing fumarate from the enzyme-malate complex and fits for the following formula.

$$\frac{k_{3F} \times \dfrac{k_{4F}}{k_{4F}+k_{4M}}}{k_{3M} \times \dfrac{k_{4M}}{k_{4F}+k_{4M}}} = 3 \qquad \text{Eq. 1.}$$

Different from aconitase, the relation between k_{3F} and k_{3M} and that between k_{4F} and k_{4M} can not be obtained respectively. The similar study to that on aconitase is performed as follows. When the substrates are mixed, (EF):(EM) = 3 (F) : 2 (M) from the ratio of two Michaelis

constants. Then the ratio of the rate of formation of malate and that of fumarate is

$$3\times3\ (F) :\ 2\times1\ (M),$$

then $4.5\ (F)\ :\ 1\ (M)$.

At the equilibrium point it is one, so

$$(M) : (F) = 4.5.$$

This value coincides with that of Krebs *et al.* (*9*) and Scott *et al.* (10), this proves the adaptability of the author's method of analysis to fumarase either. Then it can be proved that the values obtained in Section VII of RESULTS fit for Eq. 1. Different from aconitase, these values do not originate from only the ratio among k_3s, so there are possibilities of recognizing greater differences if k_3 itself can be compared. Accordingly the fact, that the ratio among k_3s is not the same in one preparation and the other is not the proof that the aconitase action is based on two kinds of enzyme. There must be a certain difference among the single enzyme itself.

SUMMARY

The evidences supporting the one enzyme and one activated-complex theory of aconitas action are added. All facts observed in the experiments carried out under vaious conditions are clearly explained by this theory.

The differences of the rate constants among different preparations are discussed. They do not deny this theory.

The reaction mechanism of fumarase is also discussed in comparison with that of aconitase.

I should like to thank Dr. H. Fukumi, Dr. D. Mizuno, and Dr. H. Uchida.

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A CONTRIBUTION TO THE KNOWLEDGE OF DEHYDRATION OF HUMAN BODY;

SOME REMARKS ON PHYSIOLOGICAL EFFECTS OF PROLONGED COMPLETE STARVATION

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Investigations on the physiological effects of dehydration have strikingly advanced our knowledge of the physiological nature of body fluid in the past three decades, and at present the significance of dehydration in diseases has come to be popularly appreciated by clinicians (see reviews of Morgulis (*1*), Rowntree (*2*), Marriott (*3*), Shohl (*4*), Darrow (*5*) and Elkinton (*6*)). Among these, however, reports on extreme dehydration in normal human subjects are comparatively rare, because the cessation of water intake makes it impossible to eat food, and brings on dangerous symptoms. On the other hand, mild or moderate dehydration is frequently observed in patients, and some information has come from observations on the complete starvation (the abstinence from water and food) of volunteers or of victims. For example, Rowntree (*2*) cited a case of an Italian political prisoner who died as the result of complete starvation for eighteen days. The drastic dehydration is believed to be the cause of death. The present knowledge on dehydration in the human body is chiefly derived from the above informations and is supplemented by those on animal experiments.

In the autumn of 1948, the authors had an oppotunity to observe Buddhist bishop, Soken Enami, 46 years, who abstained completely from water and food for 8 days in religious austerities. As such a prolonged complete starvation of a human subject is unusual, the records of the observations are believed to contribute to the knowledge of dehydration in the human body, and is thus here reported.

METHODS

Investigations were carried out on the subject occupied with religious duties in the temple, Mudo-ji, on Mount Hiei, so that the incompleteness of investigations could not be avoided because of many experimental restrictions. Thus the metabolisms mainly studied were restricted only to those of energy, water, protein and a part of salts, *i.e.* Na, K and Cl, and to the acid base balance. The physiological functions studied were respiration, circulation, temperature regulation, renal excretion etc. and the properties of blood and urine related to these metabolisms. Beside them, physical emaciation and general symptoms were also observed as far as possible.

To study the metabolism, the daily food and drink intake were measured as to their weight and composition from several days before and up to 10 days after the period of starvation. Contents of water and nutritive value of foods were calculated from " The Chemical Analysis of Food in Japan " by Saeki, and, if necessary, food was analysed chemically. Daily urine and feces were also analysed for their contents of water and nitrogenous compounds. The insensible water loss was calculated from the daily total insensible body weight loss by substracting CO_2 output and adding the oxygen absorbed. From these, the daily water and protein metabolism were computed.

Metabolisms of Na, K and Cl were investigated only from the analysis of daily urine. Na was determined by uranium acetate, K by Pt chloride, and Cl by Volhard-Salkowski's method. Acid-base balance was studied from the measurements of urinary pH, (by the quinhydrone electrode), contents of CO_2 (by Van Slyke's manomotric method), NH_3 (Folin's method) of urine and serum CO_2 (by Saito's method (*7*)). Acid-base radicals of daily urine was also titrated electrometrically (*cf.* Yoshimura (*8*)).

Daily energy expenditure was calculated from the time study on all the physical performances of the subject and their relative metabolic rates, R.M.R., described by Furusawa (*9*). The latter are the ratios of energy requirements for phyciacl performances (the total energy consumption minus the resting metabolism) to the basal metabolism, and have been used currently in Japan as the measure of intensity of muscular work. The basal and resting metabolisms were measured every day by Douglas's bag method. RM.R. of various performances, *e.g.* lying, sitting, standing, walking, sleeping *etc.* were measured before the period of starvation, and assumed to be constant during and after it. From the daily oxygen consumption and the respiratory quotient computed from the basal metabolism and energy consumption thus estimated, the three principal nutriments, *i.e.* carbohydrate, fat and protein, consumed in the body were calculated by taking into account the total nitrogen output obtained by the analysis of urine.

Regarding the physiological functions, the respiratory function was investigated from measurements of respiratory rate, ventilation volume and oxygen consumption in basal condition. The circulatory function was observed from pulse rate and blood pressure frequently measured on each day during the austerities. To investigate an outline of thermoregulatory function, the body temperature was measured twice daily at 6° *a.m.* and 6° *p.m.* and the distribution of skin temperature was measured on the nude upper half of the body at the last stage of starvation and after 10 days of rehabilitation. The latter was compared with that of the control subject measured at the same

time.

Beside these, blood was frequently drawn for determining blood cell counts, hemoglobin content (Sahli's method), water content of serum and blood (Kuroda's method (*10*)), concentrations of serum protein and blood non-protein nitrogen (half micro-Kjeldahl's method), the protein ratio of serum (from Reiss' table), viscosity (Hess' viscosimeter) and specific gravity of serum and blood (copper sulphate method), and the total circulating volume of blood (congo-red method).

Daily urine was also analysed quantitatively on its content of total nitrogen (half micro-Kjeldahl's method), creatine and creatinine (Folin's method), amino acid (the formol titration), the oxidation quotient (Müller-Kanitz's method (*11*)) with its daily total urine volume (from 6° a.m. to the same time of the next day). The qualitative tests of urine were also made on the specific gravity, protein (salicyl sulphonic acid test), sugar (Nylander's test), urobilin (Bogomolow's test), urobilinogen (with Ehlrich's reagent), aceton (Regal's test) and acetoacetate (Gerhardt's test) in daily urine.

Physical emaciation due to complete starvation was observed by measuring the daily body weight in the morning, the circumferences of chest, abdomen, neck, upper arm, thigh, and leg, the vital capacity and the grasping power, before and after the starvation.

Subjective pains and general appearance were also recorded in details, and auscultation and percussion on chest and palpation on abdomen were performed every day.

RESULTS

Outline of the Religious Austerities—The religious austerities practiced were divided into three parts. The first part was a preliminary practice of about 100 days during which the priest took daily vegetable food mainly composed of buckwheat flour, potato, and other vegetables except cereals. After this period, the complete starvation began and continued to the ninthe day (exactly for 7.6 days) (the second part of austerities). On the first day of this second period (Novem. 15), he took two meals in the morning and at noon, and thereafter ceased completely to eat and drink. When severe thirst attacked the subject, he rinsed his mouth with some water at intervals, but the water was spewed out completely after rinsing and the spewed water was ascertained by one of the authors to be rather heavier than the water used for rinsing. Even during these days of starvation, he performed his religious functions and prayed before a large sacred fire several times every day, of which the total amounted to about an hour and a half per day. Except this he retired to his bed room, and lay or slept in his bed throughout the day. On the ninth day morning (Novem. 23), he took a cup of water infused with wood pieces of *Magnolia ovovata* at first, and then "Amazake."* Thus, he took

* A Japanese hot drikts: Waxy rice granules steamed and cultured with Aspergillus oryzae were added to a rice gruel and kept at about 60° for several hours to liquify and saccharify the starch.

gradually an increasing amount of food and drink day by day, and was rehabilitated. This period of rehabilitation was the third part of the

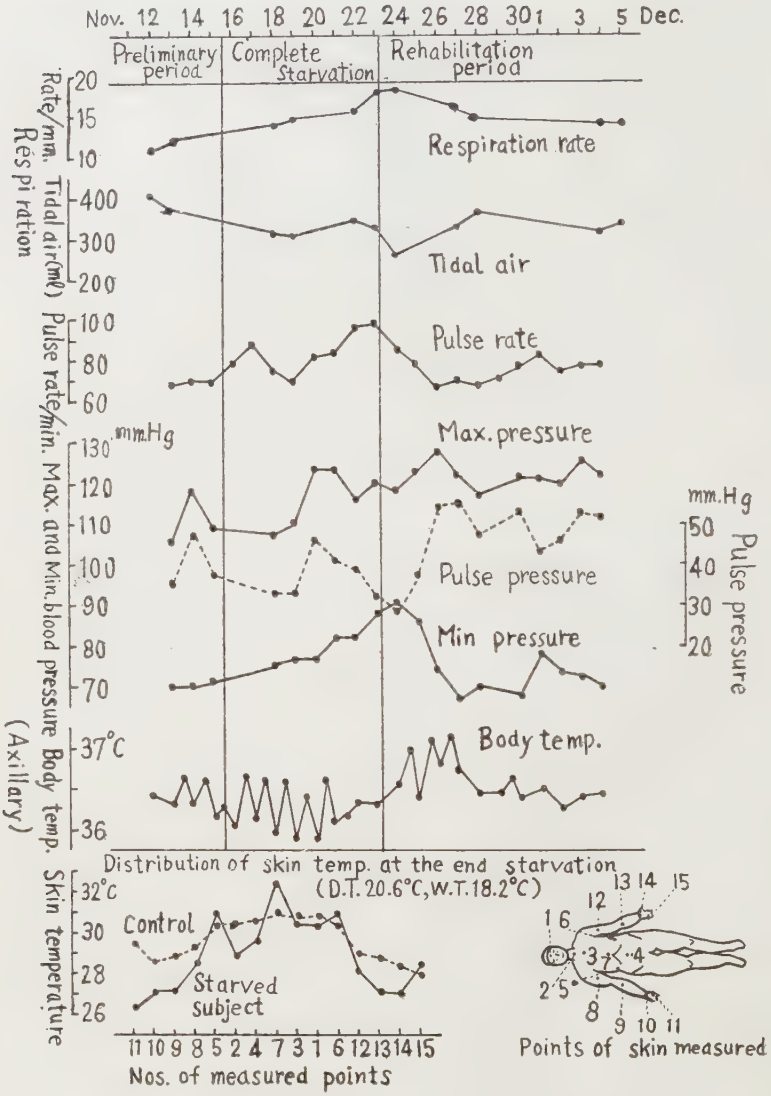

FIG. 1. Circulation, respiration and body temperature

austerities.

General Symptoms and Physical Strength—The subject suffered from severe thirst, fatigue, palpitation, dizziness *etc.* after the third or fourth day of the starvation. The general appearance was worn out and the skin was dry. Respiratory rate, pulse rate and blood pressure, especially the minimal pressure (in basal condition) rose, as the starvation progressed, while the tidal air and the pulse pressure decreased (*c.f.* Fig. 1). The rise of minimal pressure was probably caused by disturbances of peripheral blood flow due to increased blood viscosity and to contraction of peripheral blood vessels for the compensation of reduced blood volume, of which evidence was a remarkable fall of skin temperature of four limbs as described below.

Pulse and respiratory rate were accelerated more seriously by muscular exercises. For example, the pulse rate on standing changed 120/min. from 100/min. on sitting on the last day of the starvation period, while the change was insignificant before the starvation, *i.e.* to 64 from 60. Such changes were relieved considerably on the second day of the rehabilitation by the intake of water, the pulse rate being decreased to about 68 on sitting and 72 on standing. Thus the forced acceleration of circulatory and respiratory functions were mainly caused by the compensatoric mechanism for the reduction of blood volume and blood concentration. If the heart were forced to adapt to servere exercise in this condition, it might readily be exhausted. Thus, the acute heart exhaustion might be the most frequent cause of death due to dehydration.

It was worth noting that the skin of the four limbs became hypersensitive to cold when their temperature fell profoundly, and myopia developed near the end of the starvation period probably because of loss of water in the eye-balls.

The axillary temperature inclined to fall with the progress of starvation, while the basal metabolism rose somewhat (*cf.* Fig. 1 and Fig. 3). Therefore, this temperature fall should be attributed to the cooling of the skin. As the body temperature rose immediately after the restoration of circulation on the second day of rehabilitation (Fig. 1), it was supposed to be raised somewhat even in the period of starvation by the accerelated heat production and the depressed heat elimination from the skin, which were evident in the elevated basal metabolism and in the cooled limbs.

As the result of physical emaciation, theebody weight was reduced by about 15.7 per cent at the end of the starvation, and thecircum ference of various parts of the body had decreased, of which the maximum re-

duction was found on the thigh (about 20 per cent), while minimum on neck and chest (about 3 per cent) (*c.f.* Table I.).

TABLE I

Physical Examination
(Soken Enami 46 years)

Items		Preliminary period	At the last stage of starvation (Reduction in per cent)	After the rehabilitation of 2 weeks (Reduction in per cent)
Height	*cm.*	164.5	——	——
Body weight	*kg.*	51.0	43.0 (−15.7%)	49.8 (−2.4%)
Circumference of chest	*cm.*	79.0	76.2 (− 3.5%)	80.2 (+1.5%)
" of neck	*cm.*	33.4	32.5 (− 2.7%)	34.7 (+4.2%)
" of upper arm right	*cm.*	22.9	19.1 (−16.6%)	23.0 (+0.0%)
" of upper arm left	*cm.*	21.9	19.7 (−10.5%)	22.0 (+0.0%)
" of abdomen	*cm.*	76.8	65.3 (−14.7%)	74.3 (−3.2%)
" of thigh right	*cm.*	43.6	35.3 (−18.0%)	39.7 (−8.9%)
" of thigh left	*cm.*	43.8	34.7 (−20.8%)	40.3 (−8.0%)
" of leg right	*cm.*	31.7	27.3 (−13.8%)	32.1 (−1.3%)
" of leg left	*cm.*	30.7	28.5 (− 7.1%)	31.3 (−1.9%)
Vital capacity	*ml.*	2950	——	3200
Grasping power (right)	*kg.*	35.0	——	——

Blood Properties—The blood being concentrated, its specific gravity, viscosity of serum and hematocrit increased remarkably, of which the maximal value was 1.069, 2.080 and about 56 per cent, respectively. The total volume of circulating blood was also reduced by about 16.4 per cent, which was mainly due to the reduction of serum volume, *i.e.* about 36.3 per cent reduction (*cf.* Table II and Fig. 2). Such a concentration of blood necessarily caused disorders of circulation, while they were partially compensated by the marked contraction of the blood vessels of the limbs. The latter fact was shown by the mottling of limb skin and the marked fall in its temperature.

Erythrocyte and leucocyte counts, and hemoglobin and serum protein concentrations, increased also. The total amount of the latter in circulating blood decreased, however, in the last stage of the starvation, as is shown in Table II. These deficiencies of hemoglobin and serum protein were probably caused by the destructive process of body protein due to prolonged starvation. Protein quotient of serum was

TABLE II

Blood Constituents

	Averages in preliminary period	Starvation period			After taking 250 ml. of drink
		4th day	8th day	9th day	
Erythrocytes $\times 10^4$	449	489	565	——	545
Leucocytes	5440	(7200)	9100	——	9300
Haemoglobin *per cent*	83.7	93.5	107.9	——	112.1
Hematocrit *per cent*	39.3	43.4	50.0	57.3	53.3
Volume index	0.99	0.98	0.98	——	1.05
Colour index	0.94	0.96	0.95	——	1.03
Blood sp. gr.	1.0537	1.057	1.064	1.069	1.0460
Serum sp. gr.	1.024	1.026	1.032	1.032	1.0310
Spec. viscosity of serum	1.71	1.71	——	2.08	2.02
Blood water *per cent*	80.69	79.38	76.76	75.70†	76.83
Serum water *per cent*	91.85	90.78	90.04	87.24	89.45
Serum protein *g. per dl.*	5.80	6.82	9.10	9.68	9.49
Protein quotient	0.87	2.33	2.02	1.94	2.33
Total blood volume *ml.*	4017			3180*	2968
Total serum volume *ml.*	2419			1358*	1386
Total hemoglobin *g.*	471				4592
Total serum protein *g.*	147.6			131.5*	131.5
Total blood water *kg.*	3258			2.573	2.430
Total serum water *kg.*	2.218			1.221	1.278

Remarks: At the last stage of starvation, the blood sampling was very difficult owing to various experimental restrictions, and the complete examination was performed first after an intake of 250 ml. of drink. The values attached with* shows those calculated from these incomplete estimates under the assumption that the serum protein was maintained constant. † (Estimated from the blood specific gravidity).

found to increase in the starvation period.

Water Metabolism—With the progress of the starvation, water elimination from the body was reduced markedly, *i.e.* the volume of urine decreased to about 100 ml. or less per day, and the insensible water loss to about half of the normal value. Defecation was also reduced, but did not cease up to the end of the starvation, outlines of these are shown in Fig. 2. The daily water loss from various paths being summed

TABLE III

Water Loss during the Absolute Starvation of 7.6 Days.

Items			Weight	Proportion
Total loss			8,032 *g.*	*per cent* 100
Paths of elimination	Urine		2,035	25.3
	Feces		835	10.4
	Insensible loss	Skin	3,659	45.6
		Lung	1,503	18.7
Origins of water lost	Dissociation from nutriments		2,391	29.8
	Production by oxidation		1,300	16.2
	From blood water		685	8.5
	From the other tissues		3,656	45.5

up, the total amount of water eliminated from the body throughout the starvation of about 8 days was 8.03 *l.*, *i.e.* about 16 per cent of the original body weight and about 23 per cent of the total body water which was assumed to be 70 per cent of the body weight (*cf.* Table III). By assuming that the water associated with each 100 g. of carbohydrate, protein and fat in cells was 400 g. 100 g. and 20 g. respectively, the water reliesed from the tissue destroyed (total consumed nutients, in p. 369) was computed to be 2.4 *l.* and that produced by oxidation was 1.3 *l.* for the whole starvation period (see Marriott's paper (*3*)). The sum of these, *i.e.* 3.7 *l.* corresponds to the water furnished from cells during the starvation *i.e.* 46 per cent of the total loss. The loss of blood water calculated from the blood volume and the blood water contents before and after the starvation (Table II) was 0.685 *l.* corresponding to 8.5 per cent of the total loss. The remaining portion of the total water loss, *i.e.* 45.5 per cent, should be furnished from the inter- and intra-cellular free water. How much part of this may correspond to the intracellular loss, is obscure, but taking into account the above computations on intracellular water loss, it may be certain that over half of the total water lost during the starvation was furnished from intracellular water, and its proportion to the extracellular loss might not be far less than the normal proportion of the intracellular to the extracellular water, *i.e.* 5:2. Besides them,

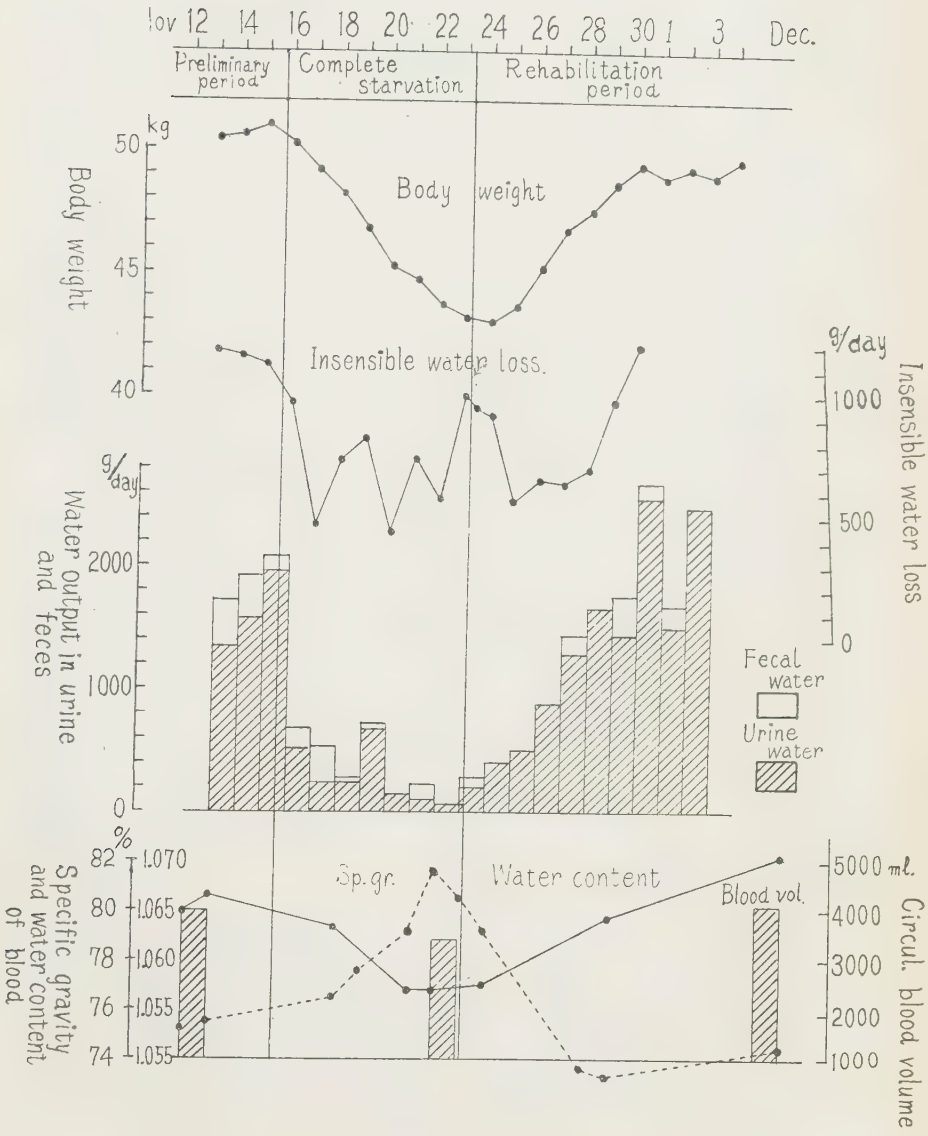

FIG. 2. Water metabolism

the proportion of blood water loss coincided nearly with the accepted percentage of normal blood water, *i.e.* about 7 per cent of total water (*cf.* Shohl (*4*)). These facts support the theory that the body acts as an osmometer as a whole (*cf.* Wolf (*12*)).

The proportions of water loss for the whole period of starvation through various paths being investigated, it was found that 36 per cent was excreted by the kidney and digestive organs, and the remaining 64 per cent corresponded to the insensible water loss (Table III). It is noticeable that more than half of the water loss was eliminated from skin and lung, and that this insensible loss was reduced to about half of its original value as the starvation proceeded, as is seen in Fig. 2. Perhaps, such a reduction owed mainly to a nearly whole day's lying in bed, and partly to some physiological changes, such as cutaneous vasoconstriction and reduced oxidation *etc.* This physical quietude of the subject's life probably enabled his body to adpat completely to this prolonged starvation without an accident, up to such stage of severe dehydration as of about 23 per cent loss of total body water.

Other Metabolic Features and Acid-base Balance—As is shown in Fig. 3, the basal metabolism rose gradually during the starvation period not only in the value per square meter of body surface, but also in the total value of the subject. Its maximum value was 38.5 Cal./m^2/hr. (1350 Cal./day) at the last stage of the starvation, and it fell gradually to the original level during the rehabilitation period. Respiratory quotient was, however, reduced during the starvation, and its minimum value was 0.72 at the end of the starvation. The fact shows that the body fat was mainly consumed as an energy source. When the food was commenced to be taken in the rehabilitation period, R.Q. rose sharply up to nearly 1.0 but did not surpass it, which fact was not in accord with Takashira's results (*13*), which showed an extraordinarily high R.Q. after the starvation.

From daily energy expenditure estimated by the time study, daily urine nitrogen (see below) and non-protein R.Q. estimated in the basal condition, the bodily nutriments daily consumed were calculated of which caloric proportions are shown in histograms of basal metabolism in Fig. 3. It is shown that the carbohydrate was burned off already on the third day of the starvation. Total weights of consumed nutriments calculated for the total starvation period were 437 g. of carbohydrate,

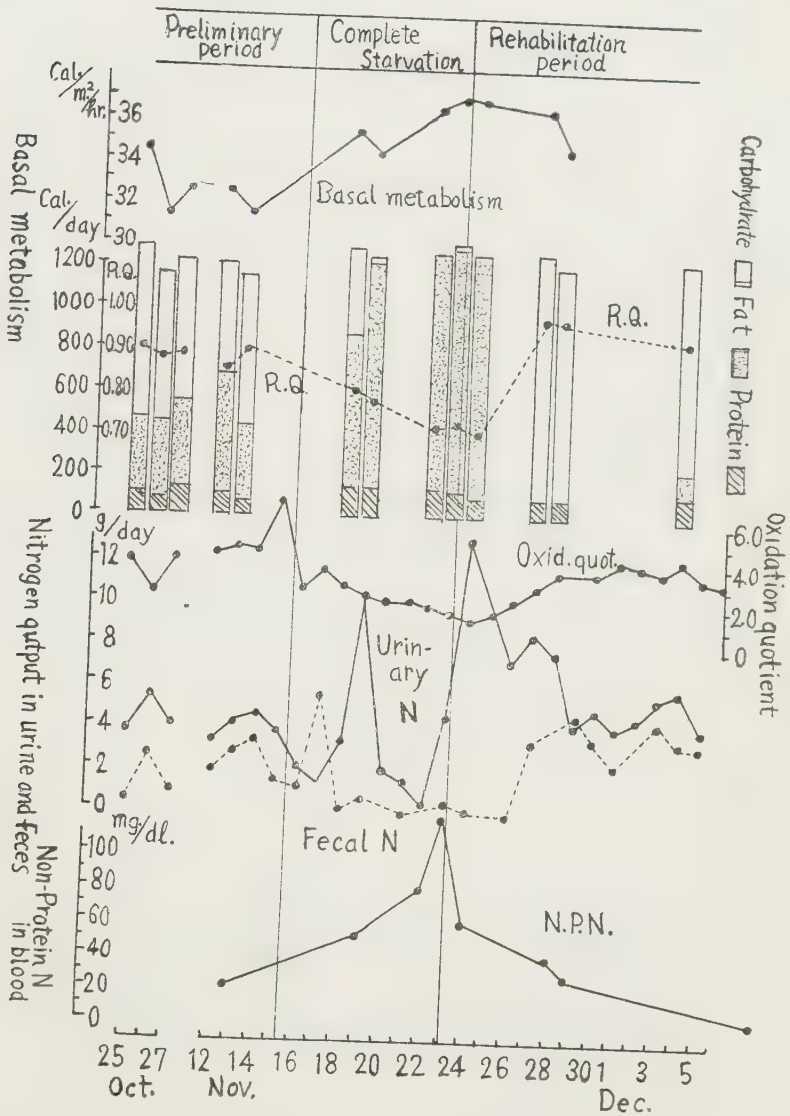

FIG. 3. Energy and protein metabolism

896 g. of fat and 305 g. of protein.*

On the protein metabolism, a more detailed explanation is necessary. As is shown in Fig. 3, the daily nitrogen out-put in urine was decreased in the starvation. This reduction was probably not due to the decrease of protein consumption, but to the retention of urinary constituents in the blood as non-protein nitrogen by the reduction of urinary excretion, for the nitrogen out-put in urine increased and non-protein nitrogen in the blood decreased immediately, when the urine volume was restored by a sufficient water intake in the beginning of rehabilitation. Another evidence for this explanation was a sharp rise of urinary nitrogen due to an accidental increase of urinary volume on the fourth day of the starvation (see Figs. 2 and 3.), due to a slight emotional excitement of the subject. It is interesting that the water loss was increased, and the blood pressure, especially the pulse pressure was raised and physical weariness was increased on the next day to this minute accident (*cf.* Figs. 1 and 2). The fact suggests that the mental quietude was also necessary for adaptation to starvation.

As the total consumed nutriments originated from the tissue destruction, the increased tissue metabolites necessarily caused acidosis in the starvation period (Fig. 4). The reaction of urine shifted markedly to the acid side, acetone bodies were detected and the concentration of ammonia and the base economy increased. As is shown by the same figure, the alkali reserve in the blood was also reduced to some extent, though not so makedly. The creatine excretion increased and the urobilinogen reaction with Ehlrich's reagent was strongly positive.

Excretion of Na, K and Cl in urine was reduced, and the ratio of N/K increased gradually with the progress of starvation, which fact

* The consumption of protein during the starvation was calculated from the sum of the urinary output of nitrogen during the starvation period, and that of its excess nitrogen eliminated from the raised non-protein nitrogen in the body during the early period of rehabilitation (about 5 days after the end of the starvation as was shown in Fig. 3.) To calculate the latter, the basal daily output of urinary nitrogen throughout the whole rehabilitation period was assumed to be the same as that of its later period when the non-protein nitrogen in the blood was restored to its normal limit, *i.e.* after the sixth day (Novem. 29th) of the rehabilitation. This basal output was 4.82 g./day on an average, and thus the total excess output of nitrogen during the first five days of the rehabilitation was calculated to be 22.8 g. As the sum of nitrogen output during the starvation period was 26.0 g. the total nitrogen derived from the protein consumed in the body during the starvation was 48.8 g. which corresponded to about 305 g. of protein.

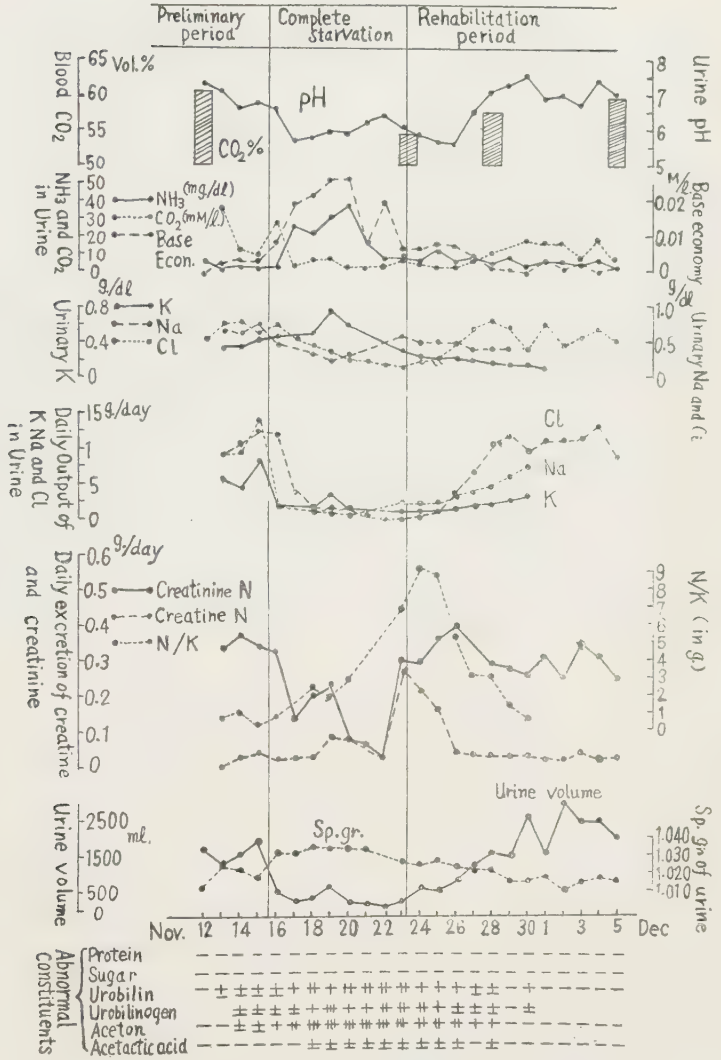

FIG. 4. Acid-base balance and urinary excretion

was probably due to an increase of reabsorption of K in the kidney (Fig. 4). Thus the urinary excretion was so adapted as to reduce the elimination of water with the important fixed bases useful for the body.

SUMMARY

From observations on a Buddhist priest who abstained completely from food and water for about 8 days, the following conclusions were drawn.

1. The subject lost about 23 per cent of total body water (about 8 litres) after about 8 days starvation, of which over half corresponded to the insensible water loss, though the latter was also adapted to reduce during the starvation to about half of its normal value.

2. Over the half of the lost water was supplemented in the body by the liberation of intracellular water which was mainly associated with nutriments and thus the proportion of intracellular water loss to the total loss was approximately the same as its normal value in the body. The same can be said on the blood water loss. It was presumed that the body acted approximately as an osmometer as a whole.

3. While the circulating blood volume was reduced by about 16 per cent, blood vessels contracted especially in the four limbs for compensation and thus the circulation was maintained. In muscular exercise, however, the circulation and the respiration were forced to accelerate, and the heart was threatened with exhaustion to overcome circulatory disturbances due to blood concentration. Such acute heart exhaustion may presumably be the main cause of death ue to dehydration.

4. The basal metabolism was raised to some extent probably by the concentration of body fluids, and the body temperature rose a little, though the axillary temperature was lessened by the cooling of skin temperature.

5. Adaptative changes of various physiological functions and metabolisms were described together with general symptoms accompanying absolute starvation.

6. It was found that the mental and physical quietude was an essential factor for the adaptation to severe dehydration or absolute starvation.

The author wish to express their cordial thanks to Buddhist Bishop, Soken Enami for his kindness to permit the investigations on his body, and also to priests in the temple, Mudo-ji, for their kind assistances.

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STUDIES ON NON-HEMIN IRON

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(Received for publication, April 20, 1953)

Biological iron compounds can be chemically classified into the following two groups: I) Hemin compounds, such as hemoglobin, cytochrome, catalase and peroxidase, whose chemical nature has been studied well; and, II) Non-hemin iron compounds, which play an important rôle in iron metabolism, but whose chemical nature has not been studied in detail except with ferritin. Although ferritin is a comparatively wellstudied fraction of non-hemin iron compounds, we have only immunochemical methods for its quantitative determination at present.

In the present investigation the non-hemin iron compounds were separated into four fractions, and the iron content of each fraction especially of ferritin, was determined quantitatively.

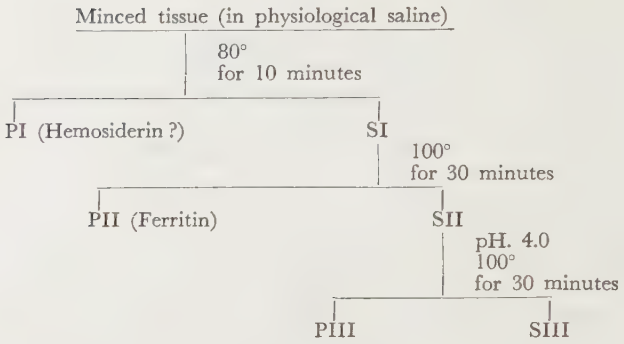
EXPERIMENTAL METHODS AND RESULTS

I. The Method of Fractionation—Ferritin is soluble in water at all temperatures between 0° and 80° and is precipitated from the solution at 100°. This characteristic property was used for the fractionation.

A suspension of minced tissues (0.5–2.0 g. of liver, spleen, and kidney) in 5 ml. of physiological saline solution was heated to 80° in a water bath for 10 minutes, and then centrifuged to obtain a precipitate, designated as PI. The supernatant (SI) was removed to another tube, and the precipitate was washed two or three times with physiological saline. Thus the minced tissue was separated into saline-soluble and saline-insoluble fractions. The supernatant (SI) placed in a tube was heated again at 100° for 30 minutes. The precipitate obtained at this stage was designated as PII. Acetate buffer was added to the supernatant (SII) and its pH was brought to 4.0. The solution was heated again at 100° for 30 minutes and centrifuged. The precipitate and the supernatant obtained at this stage were respectively designated as PIII and SIII. Fig. 1 shows the outline of the fractionation.

Non-hemin iron of all these fractions was determined according to the method of Brueckmann and Zondeck (*1*) as follows: Three to four ml. of the mixed solution of the equal amounts of 10 per cent trichloroacetic acid and 4 per cent sodium pyrophosphate were added to each fraction and the mixture was heated at 100° for 30

FIG. 1.



minutes. As non-hemin iron was known to be extracted by this solution, the mixture was centrifuged and the supernatant was removed to another tube. The same procedure was repeated tow or three times, and the supernatants were combined in one tube and neutralized with ammonia, using phenolphthalein as an indicator. Acetate buffer was then added to this solution to bring its pH to 4.0, and one drop of thioglycol-

TABLE I

Amount of Iron in PI, PII, PIII and SIII of Rat Organs
(Unit is γ per 1 g. wet weight of organ)

	Liver				Spleen				Kidney			
No. of animal	PI	PII	PIII	SIII	PI	PII	PIII	SIII	PI	PII	PIII	SIII
1	96	52	21	4	164	70	10	6	67	12	7	1
2	58	64	22	3	320	40	48	3	14	10	5	1
3	30	45	10	4	48	59	36	0	18	9	18	3
5	44	69	12	2	21	60	11	0	14	11	9	0
10	62	52	8	1	24	9	12	1	37	8	13	0
13	48	31	5	1	70	29	7	3	68	12	12	3

lic acid, 1 ml. of saturated phenanthroline solution, and water were added to make its volume to 25 ml. Iron content was determined from the extinction of the color developed, using the standard curve derived from the solution of Mohr's salt.

The results are shown in Table I. In liver, the non-hemin iron of PI, PII, PIII and SIII does not fluctuate so much, but in spleen, the amounts of PI, PII, PIII, and SIII fluctuate remarkably, owing

perhaps to the condition of the spleens.

II. The Determination of Ferritin Iron by the Antigen-Antibody Reaction—

a) *Antigen*—Ferritin was crystallized according to the Mechaelis method (*2*) from the livers and the spleens of 60 dogs. The crystalline preparation was dissolved in physiological saline to make the 0.5 per cent solution.

b) *Antibody*—The antigen was injected for 4 or 5 weeks into 5 rabbits from ear veins 2 or 3 times a week. When the titer of the antibody of the rabbit serum became high, the animals were killed by bleeding and the sera were used as antisera.

c) *Antigen-Antibody Reaction*—One ml. of SI mentioned above was added to 1 ml. of the antiserum, and the mixture was kept at 37° for 1 hour and then placed at 2 to 4° overnight. The precipitate obtained by centrifugation was washed with cold saline, and the non-hemin iron of the precipitate was determined in the same manner as mentioned in Experiment 1.

The results are shown in Table II. The iron content of PII agreed almost with that of ferritin-iron determined by this antigen-antibody reactions. The values of the former were only 2–3 per cent higher than the values of the latter.

TABLE II

Comparison of the Values of Ferritin Iron of Dog Organs Obtained by Chemical Method and by Immunological Method

(Unit is γ per 1 g. wet weight of organ)

Organ	Liver							Spleen						
No. of animal	17	18	19	1′	17′	18′	19′	17	19	17′	18′	19′	15	1′
Chemical	57	69	85	82	50	21.3	44.5	55	105	30	22	42.5	66	84
Immunological Method	53	65	82	77	50	20	42	53	100	28	21	44.5	61.5	81

III. Recovery Test—To ascertain that the fraction PII was really ferritin fraction, the recovery test was carried out in the following way. The fractionation described in *Section I* was carried out on the following three specimens. The first and the second were a mixture of 1 ml. of the horse liver homogenate, and 1 and 2 ml. of the recrystallized horse ferritin solution respectively, which contained 19γ of iron per ml., while the third specimen contained the homogenate only. The results of duplicate experiments are shown in Table III.

DISCUSSION

PI in the above experiment is the fraction which can not be ex-

TABLE III

Recovery Test

Amount of iron content: The first and the second specimens were a mixture of 1 ml. of the horse liver homogenate, and 1 and 2 ml. of ferritin solution, respectively. The third specimen contained the homogenate only.

Specimen No.	Ferritin added.	Homogenate.	
1	19 γ	24 γ	24 γ
2	38	43	43
3	0	4	4

tracted with physiological saline, and it seems to consist mainly of the so-called hemosiderin. Ferritin does not coagulate at 80° and it is easily soluble in saline, so that the PI fraction does not contain ferritin. The large percentage of the total non-hemin iron is contained in PI in liver, spleen and kidney, especially in spleen. This fact agrees with the histological observation which indicates that hemosiderin is usually present in spleen. It must be of significance to study this PI fraction, because it seems to play an important part in the iron metabolism.

PII, which is precipitated by heating at 100° is mainly composed of ferritin. The iron in this fraction was proved to come really from ferritin by the antigen-antibody reaction and by the recovery test using liver homogenate. So this PII fraction can be regarded as the ferritin fraction. By measuring iron content of this fraction, we can determine the ferritin-iron, which has hitherto been determined only by antigen-antibody reaction.

PIII, which is precipitated by heating at 100° for 30 minutes at pH 4.0, is considered to contain the iron bound to nucleoprotein and other high molecular compounds which are not precipitated with PI and PII. The amount of iron in this fractions is very small but seems to be constant.

The fraction SIII does not contain high molecular compounds, and thus the iron in this fraction is considered to be free iron, but the amount of iron in this fraction is very small.

Amoung the tested organs, that is, liver, spleen and kidney, spleen was found to be the richest in non-hemin iron. This fact is noteworthy because the spleen is an important organ for the destruction of red blood cells.

SUMMARY

1. Non-hemin iron of rat liver, kidney and spleen was separated into four fractions.

2. The PI fraction probably contains hemosiderin. In spleen, PI showed the highest value of iron content among the four fractions. This indicates that there are much hemosiderin in spleen.

3. Iron in the PII fraction was proved to come really from ferritin by the antigen-antibody reaction and by the recovery test. The liver was rich in this fraction.

4. Iron content of the PIII fraction, which is attributable to nucleoprotein and the high molecular compounds, was very small.

5. The so-called free-iron was present only in a small amount or was not present in the tissues studied.

6. Among spleen, liver and kidney, spleen was found to be the richest in non-hemin iron.

The authors express their cordial thanks to Prof. N. Shimazono, Prof. H. Yo-Yoshikawa and Prof. K. Kodama for their kind guidance.

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STUDIES ON XANTHURENIC ACID

IV. EFFECT OF INSULIN ON TRYPTOPHAN METABOLISM

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(Received for publication, April 23, 1953)

In the foregoing papers of this series of study it has been reported that when sodium salt of fatty acid and tryptophan are administered together to rat following the prescriptions of Y. Kotake (Jr.) and Inada, xanthurenic acid is abundantly excreted into urine (*1*), and the animal shows pancreas diabetic symptoms (*2*). Further it was tentatively assumed that the administration of a large amount of fatty acid caused an acute B_6-defficiency in rat as suggested by Lepkovsky (*3*) and this defficiency led to an abnormal metabolism of tryptophan to xanthurenic acid.

In this case it seems to be interesting to investigate how far insulin can correct this abnormal situation of amino acid metabolism, since insulin has a direct bearing on the genesis of diabetes and further some concerns on the protein metabolism as depicted by Forker and Chaikoff (*4*).

The experimental result, which will be reported here, substantially proves that insulin can reduce the output of xanthurenic acid following the ingestion of test dosis of tryptophan and butyrate and can increase the excretion of anthranilic acid, which is a normal metabolite of tryptophan.

EXPERIMENTAL

To one group of white rats weighing 150 g., 0.1 g. of tryptophan and 0.4 g. of sodium butyrate were ingested, and to another group of rats similarly treated a certain dosis of insulin was injected. On the next day 24 hours urine was collected from each animal and treated following the method of Neuberger *et al* (*5*). Namely, a sufficient amount of mercuric acetate was added to urine to cause maximal precipitation and

left overnight. The precipitate was collected, suspended in water, decomposed by hydrogen sulfide and filtrated. The filtrate was condensed to 1.0 ml. at reduced pressure avoiding rise of temperature and then subjected to chromatographic analysis using Toyo filter paper No. 2. The developing mixture consisted of butanol, acetic acid and water (4:1:1), and the developing time was 15 hours. The result is indicated in Fig. 1 where the spots detected by fluorescence test are shown. Further on the extracts from each spots various qualitative chemical tests were carried out, the results of which are given in Table I together with their Rf-values.

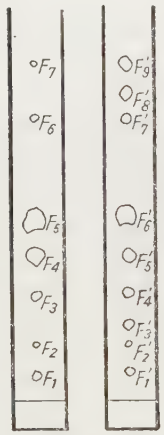

FIG. 1. Paper chromatography of urine constituents (spots detected by fluorescence) S: Urine from control rats. I: Urine from rats injected with insulin.

It is almost sure that the spot F_5 corresponds to F'_6 and is caused by xanthurenic acid on the basis of various chemical tests, and further by the finding that both spots became more enlarged and intensified when pure xanthurenic acid was added previously to urine. But it should be noticed that F'_6 gave in every case apparently weaker xanthurenic acid reactions than F_5, such as the Ehrlich's, the Pauly's, the Millon's and Fe^{+++} color reactions. It might be concluded, therefore, that insulin hinders the production of xanthurenic acid. With regard to other spots F_7 and F_9' correspond to each other and are identified as due to anthranilic acid. In all samples tested, however, F'_9 gave more intense reaction than F_7. Since anthranilic acid is considered to be an usual metabolite of tryptophan, it will be assumed that insulin has an ability to regulate the path way of tryptophan metabolism into the normal one under the condition of this experiment.

There seems to be no parallelism between the dosis of insulin and the reduction of xanthurenic acid output. With the expectation that a larger dose of insulin might reduce xanthurenic acid almost to nil, various doses of insulin were tried. But this was not the case as shown in Fig. 2. In the course of the above mentioned study we came to isolate a compound (about 10 mg. from each sample of 24 hours urine). This compound gave the positive Kretchy's and Jaffe's tests and melting point at 268–9°. These properties coincide with those of kynurenic acid. To raise futher evidence its ultra violet spectrum was taken by means of

TABLE I

Chemical Tests of Urine Constituents

Color reaction	S					I					
	F_3	F_4	F_5	F_6	F_7	F'_4	F'_5	F'_6	F'_7	F'_8	F'_9
Rf. value	0.32	0.43	0.50	0.71	0.89	0.32	0.43	0.50	0.71	0.80	0.89
Bratton-Marshall	−	+	−	+	+	−	+	−	+	+	+
Ehrlich	+	−	+	−	−	+	−	+	−	−	−
Pauly	+	−	+	−	−	+	−	+	−	−	−
Millon	−	−	+	−	−	−	−	+	−	−	−
Fe^{+++}	−	−	+	−	−	−	−	+	−	−	−
Ninhydrin	+	+	−	−	−	+	+	−	−	−	−

S: Urine from confrol rats, I: Urine from rats injected with insulin.

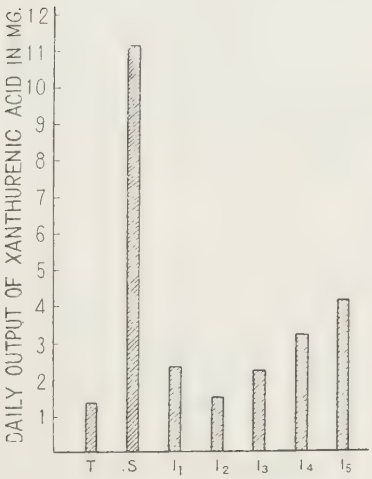

FIG. 2. Effect of insulin on the excretion of xanthurenic acid.

T: Animal which received tryptophan, S: Animal which received tryptophan and fatty acid, I: Animals which received tryptophan and fatty acid, injected with inssulin in the following doses: I_1 0.05 U., $I_2$0.1 U., I_3 0.2 U., I_4 0.5 U., I_5 1.0 U.

Beckman photometer. The result is illustrated in Fig. 3, where spectrum of pure kynurenic acid is also indicated for comparison. Fig. 3 clearly demonstrates that the compound is kynurenic acid.

SUMMARY

1. Insulin can supress the excretion of xanthurenic acid in rat ingested with tryptophan and sodium butyrate. Instead, it increases

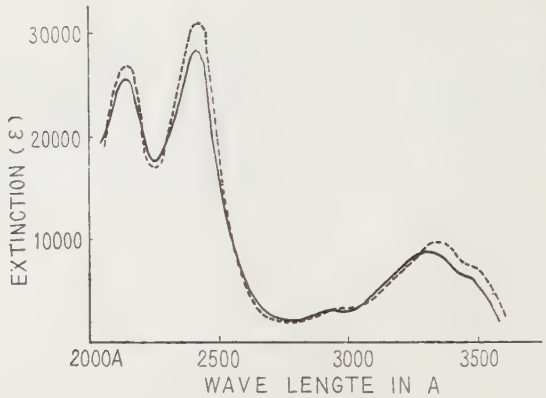

FIG. 3. Ultraviolet spectrum of a conpound from urine, compared with kynurenic acid

——: Sample; - - - -: Kynurenic acid (Roche Co.)

the output of anthranilic acid.

2. From urine of rat ingested with tryptophan and sodium butyrate followed by the injection of insulin, a fairly large amount of kynurenic acid was isolated.

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MECHANISM OF MUSCULAR CONTRACTION‡

II. KINETIC STUDIES ON MUSCLE ATP-ASE‡‡

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(Received for publication, May 4, 1953)

Since the discovery of the splitting of ATP in the presence of actomyosin, and of the deformation of actomyosin particles by the addition of ATP, various attempts have been made to explain the muscular contraction in terms of the interaction of actomyosin with ATP.

In the previous paper (*1*), it was shown that the change in light-scattering of actomyosin solution caused by addition of ATP can be divided into three steps as follows:

First step: $AM+S \rightleftarrows AMS \longrightarrow AM^*S$‡‡‡

Second Step: $AM^*S \longrightarrow AM^*+P$

$AM^*+S \rightleftarrows AM^*S$

Third step: $AM^* \longrightarrow AM$.

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‡ Supported in part by a grant from the Scientific Research Funds of the Ministry of Education.

‡‡ In this paper, the following abbreviations are used: ATP=adenosine triphosphate, ADP=adenosine diphosphate, M=myosin, AM=actomyosin, P=inorganic ortho-phosphate (or plus ADP).

‡‡‡ In the previous paper (*1*), the following mechanism was proposed:

$$AM+S \longrightarrow AMS \longrightarrow AM^*S$$
$$\searrow AM+P$$

The modification adopted in this paper is believed to be better based on the fact that the speed of the first step is not influenced by the addition of calcium ions (*cf.* (*1*) Fig. 13a.). Corresponding to this change of our picture, parts of Table VI in the previous paper must be corrected as follows:

Reaction	Velocity Constant	
$AM+S \longrightarrow AMS$	${}_1k={}_{Ca}k={}_{Mg}k=$	10×10^4 (*lit./mole sec.*)
$AMS \longrightarrow AM+S$	${}_1k={}_{Ca}k=2$	(*1/sec.*)

That is, the binding reaction of actomyosin with ATP is not affected by calcium and magnesium ions.

In this scheme, AM is a component unit of actomyosin which contains 140,000 gm. of myosin and its affix * represents the deformed state: then S and P stand for the substrate of the ATPase, *i.e.*, ATP plus H_2O and the products of enzymatic hydrolysis of ATP, *i.e.*, ADP plus inorganic phosphate, respectively. Further, our kinetic study of ATPase action (Second step) in the presence of ca. 0.2 *M* KCl revealed that the action is inhibited by Mg^{2+} and is activated by Ca^{2+} and that the relation of the ATP concentration to the reaction rate obeys the well known Michaelis-Menten's formula.

In this paper are presented further experiments on the kinetics of myosin-ATPase and natural actomyosin (myosin B)-ATPase.

EXPERIMENTAL PROCEDURES

Preparation of Materials—ATP and myosin B (actomyosin) were prepared as previously described (*1*). Purified myosin was prepared according to Szent-Gyorgyi's method (*2*).

Determination of ATPase Activity—The enzyme reaction was started by adding 0.5 ml. of K-ATP solution to 2.5 ml. of the enzyme solution containing salts and buffers as to give the final concentration of salts and pH value desired (the concentration of the enzyme in the reaction mixture was about 0.1∼0.3 mg. protein per ml.). At certain intervals, the reaction was stopped by adding 1.0 ml. of 10 per cent trichloracetic acid. Other procedures were identical with those described in the previous paper (*1*).

EFFECTS OF MAGNESIUM AND CALCIUM IONS ON ATP-ASE ACTION

It is well known that the effects of divalent ions, especially that of Mg^{2+}, on the muscle ATPase are greatly modified by the KCl concentration (3). In the present work, the effects of Mg^{2+} and Ca^{2+} on actomyosin-ATPase were investigated its relation to the level of KCl concentration in the reaction medium and also the antagonism between Mg^{2+} and Ca^{2+} was studied. The concentrations of ATP used in these experiments were about 10^{-3} *M*, taking acount on the situation that the activity of ATPase was approximately independent of the ATP concentration at about 10^{-3} *M*.

Effects of Calcium Ion—As can be seen in Fig. 1 a-b, Ca^{2+} strongly enhances the ATPase actions of both actomyosin and myosin in the presence of all concentrations of KCl investigated. The ratio of the velocity of ATPase action in the presence of less than 10^{-2} *M* Ca^{2+} (v_{Ca}) to that of the control (in the absence of Ca^{2+}) (v_K) is given by the

equation:

$$\frac{v_{Ca}}{v_K}=1-\frac{1-\Delta_{Ca}}{1+\frac{K_{Ca}}{[Ca]}}.$$

This means that the ATPase activity of actomyosin (or myosin) unit increases to Δ_{Ca} times that of the control on binding with one mole of Ca^{2+} and the dissociation constant for this binding reaction is K_{Ca}. For example, Δ_{Ca} and K_{Ca} of actomyosin (myosin B)-ATPase at pH 9.2, 10°, in the presence of 0.15 *M* (K^++Na^+), are 13 and $10^{-2.4}$ *M* respectively (Fig. 1a). Hereafter, the binding point of myosin and actomyosin responsible for the combination with Ca^{2-} in question ($pK=2.4$) will be termed " the first ion-binding point " and the resulting Ca-complexes will be denoted by Ca(I)-M and Ca(I)-AM*. A relationship of the ATPase activity of Ca(I)-AM* to the KCl concentration is roughly shown in Fig. 2.

In the presence of Ca^{2+} in concentrations higher than about 5×10^{-2} *M*, the ATPase activity decreases with the increase of the Ca^{2-} concentration. This inhibitory effect is not discussed in this paper.

Effects of Magnesium Ion and Antagonism between Magnesium and Calcium Ions at Higher K-concentrations—As already reported by Banga *et al.* (3),

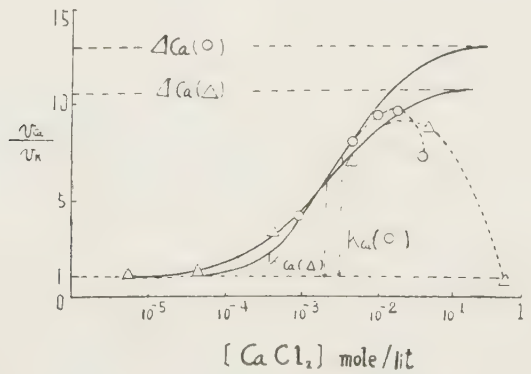


Fig. 1a. Effect of calcium ion on actomyosin-ATPase
(○) pH 9.2 (glycine), 10°, [K^+]=0.15 *mole/lit.*,
[ATP]=1×10^{-3} *mole/lit.*
(▲) pH 6.5 (veronal-acetate), 22°,
[K^++Na^+]=0.08 *mole/lit.*,
[ATP]=1.17×10^{-3} *mole/lit.*.

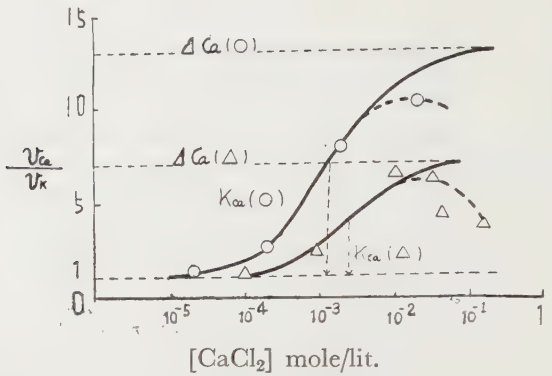


Fig. 1b. Effect of calcium ion on myosin-ATPase
(○) pH 6.6 (veronal-acetate), 20.5°
$[K^+ + Na^+] = 0.073$ *mole/lit.*
$[ATP] = 1.67 \times 10^{-3}$ *mole/lit.*
(△) pH 7.0 (veronal-acetate), 20°
$[K^+ + Na^+] = 0.077$ *mole/lit.*
$[ATP] = 1.69 \times 10^{-3}$ *mole/lit.*.

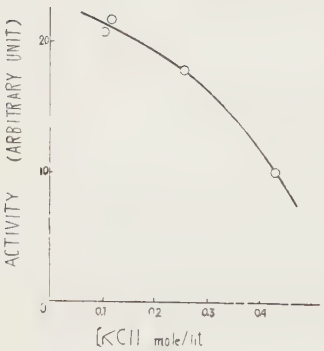


Fig. 2. Effect of the KCl concentration on actomyosin-ATPase in the presence of calcium ion. pH 6.5 (veronal-acetate), 18.7°

the effects of Mg^{2+} on actomyosin-ATPase are strikingly influenced by the concentration of KCl. In the presence of KCl over 0.1 *M*, the ATPase action of actomyosin is inhibited by Mg^{2+} and, as will be seen in Fig. 3a, the ratio of the velocity of its ATPase action in the presence of varied $MgCl_2$ concentrations less than 10^{-3} *M* (v_{Mg}) to that of the control (v_K) is approximately given by:

$$\frac{v_{Mg}}{v_K} = 1 - \frac{1-\Delta_{Mg}}{1+\frac{K_{Mg}}{[Mg]}}$$

where Δ_{Mg}, unlike Δ_{Ca} described above, is very much smaller than unity. That is, the ATPase activity of an actomyosin unit is depressed to Δ_{Mg} times that of the control on binding with one mole of Mg^{2+} and the dissociation constant for the binding of actomyosin (or myosin) with Mg^{2+} is K_{Mg}. For example, Δ_{Mg} and K_{Mg} at pH 9.2, 10°, in the presence of 0.15 *M* ($K^+ + Na^+$) are 0.2 and $10^{-4.4}$ *M*, respectively (Fig. 3a).

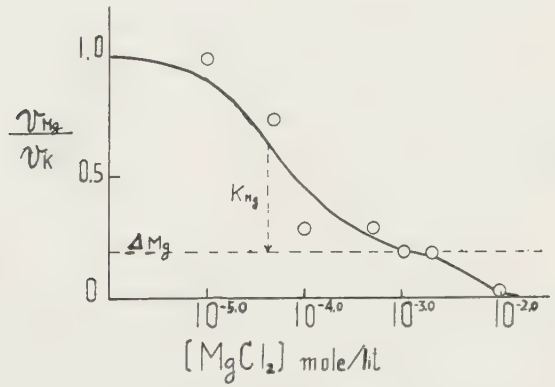


FIG. 3a. Effect of magnesium ion on actomyosin-ATPase (I).
pH 9.2 (glycine), 10°, $[K^+]=0.15$ *mole/lit.*
$[ATP]=1\times10^{-3}$ *mole/lit.*.
The fact that 10^{-2} *mole/lit.* Mg^{2+} caused stronger inhibition than 10^{-3} *mole/lit.* Mg^{2+} may be supposed to be due to the formation of Mg (I)Mg(II)-AM.* (*cf.* Page 393)

In the presence of a certain concentration of Ca^{2+} ($[Ca^{2+}] \gg K_{Ca}$), addition of Mg^{2+} results in an inhibition of ATPase activity. This inhibition was investigated in detail in the foregoing work (*1*) and found to be competetitive with regard to Ca^{2+} and Mg^{2+}. In this case (see (*1*) Fig. 5, 6), a similar equation to that described above may be formulated approximately:

$$\frac{v_{Mg,Ca}}{v_{Ca}} = 1 - \frac{1-\Delta_{Mg,Ca}}{1+\frac{K_{Mg,Ca}[Ca]}{[Mg]}}$$

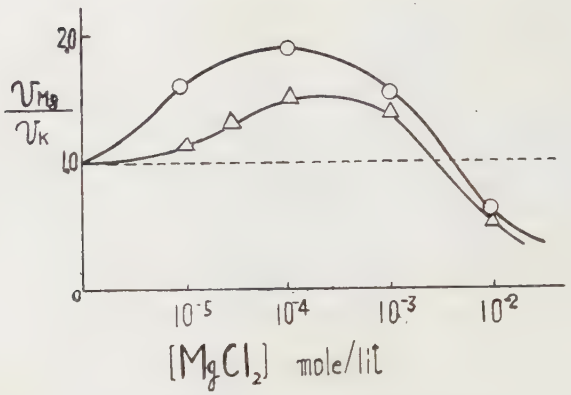


FIG. 3b. Effect of magnecium ion on actomyosin-ATPase (II)
pH 6.5 (veronal-acetate)
$[K^+ + Na^+] = 0.08$ *mole/lit.*
$[ATP] = 1.17 \times 10^{-3}$ *mole/lit.*
24° (○) and 20° (△).

Here, $\Delta_{Mg, Ca}$ in the presence KCl over 0.1 *M* is very much smaller than 1 and $K_{Mg, Ca}$ at pH 9.2, 10° in the presence of 0.15 *M* ($K^+ + Na^+$) is 10^{-2} *M* which is equal to the ratio of K_{Mg} ($10^{-4.4}$) to K_{Ca} ($10^{-2.4}$) under the identical experimental conditions as derived from the experiments on the Mg-inhibition (Fig. 3a) and Ca-activation (Fig. 1a). The latter fact suggests that the Mg^{2+}-binding point of actomyosin with $pK = 4.4$ is identical with the first Ca^{2+}-binding point with $pK = 2.4$ mentioned above.

Effect of Magnesium Ion and Antagonism between Magnesium and Calcium Ions at Lower KCl-Concentrations—As shown in Fig. 3b, in the presence of KCl less than 0.1 *M*, addition of Mg^{2+} ($10^{-5} \sim 10^{-3}$ *M*) stimulates the ATPase action of actomyosin‡ but an inhibitory effect is observed when the concentration of Mg^{2+} is increased to 10^{-2} *M*. These facts are probably due to the situation that, in these concentrations of KCl, the enzymatic activity of Mg(I)-actomyosinate is stronger than that of K-actomyosinate (about 1.5~2 times) and, when another mole of Mg^{2+} combines with Mg(I)-actomyosinate (the dissociation constant of this binding is about $10^{-2.5}$), the enzymatic activity of Mg-acto-

‡ The magnitude of this activation depends not only upon the KCl concentration but also on the preparative conditions of enzyme which are uncontrollable for us.

myosinate becomes less than that of K-actomyosinate (about one half that of K-actomyosinate). This Mg^{2+}-binding point of actomyosin with $pK=2.5$ will be termed "the second ion-binding point." As can be seen in Fig. 4, the pH-activity curve of Mg(I)-actomyosinate has one maximum at about pH 7.5 whereas that of Ca(I)-actomyosinate has been shown to have two maxima at pH 6.3 and 9.7 (see (*1*) Fig. 4).

Now, it is very interesting that, in the presence of 10^{-2} *M* Mg^{2+}, a concentration enough to set all the AM molecules in combination with Mg^{2+} (Mg(I)Mg(II)-AM*), the addition of a small amount of Ca^{2+} (10^{-3} *M*) causes the increase of the enzymatic activity of actomyosin as shown in Fig. 5.# It is unlikely that this effect is caused by the partial substitution of Mg^{2+} in the first ion-binding point by Ca^{2+} because the ratio of $[Ca^{2+}]$ to $[Mg^{2+}]$ (1/10 in this experiment) is as low as about one-thousandth of the ratio of the dissociation constants for the Ca^{2+} binding to that for the Mg^{2+} binding with the first ion-binding point (60∼180, see Figs 1a, 3a). Further, as shown in Fig. 5, the ATPase activity is unchanged over the wide rage of Ca^{2+} concentrations added ($10^{-3}\sim 3\times 10^{-2}$ *M*) and the activity begins to rise when the Ca^{2+} concentration increases to such height (more than 10^{-1} *M*) that the substitution at the first ion-binding point is expected to take place. Hence, the above effect is probably derived from the conversion of Mg(I)Mg(II)-actomyosinate## into Mg(I)Ca(II)-actomyosinate as the affinity of Ca^{2+} to the second ion-binding point (in contrast with that to the first ion-binding point) is much stronger than that of Mg^{2+}.### Similar effects are observed also in the presence of 0.15 *M* KCl and the activity of Mg(I)Ca(II)-AM* is, in this case, about seven times stronger than that of Mg(I)Mg(II)-AM.*####

This activation is markedly dependent upon uncontrollable conditions of the enzyme preparations. The slight inhibition is occasionally observed. The investigation of this point must be the subject of the future research.

Mg(I)Mg(II)-actomyosinate represents the actomyosin combined with Mg^{2+} in both first and second ion-binding points. If the similar expression is to be applied to the already mentioned Ca-actomyosinate or Ca(I)-actomyosinate, it should be expressed as Ca(I)Ca(II)-actomyosinate.

Since the addition of 10^{-3} *M* Ca^{2+} in the presence of 10^{-2} *M* Mg^{2+} causes the complete substitution of Mg^{2+} in the second ion-binding point by Ca^{2+}, it follows that the dissociation constant for the binding of Ca^{2+} with the second ion-binding point is sufficiently smaller than $(10^{-2.5}\times 10^{-3}/10^{-2}=)\ 10^{-3.5}$.

In this case, the activity of Mg(I)Ca(II)-AM* is high as compared with that of Mg(I)Mg(II)-AM* but is only about one-fifth of that of K-AM*.

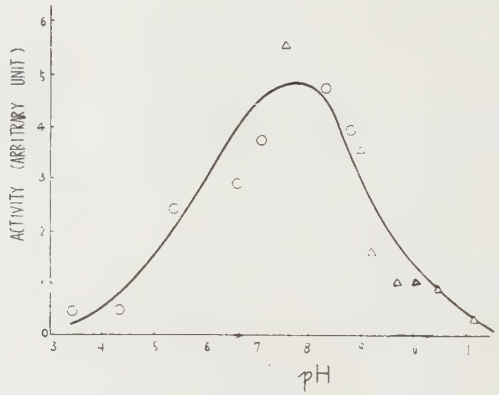


FIG. 4. pH-activity curve of Mg^{2+}-activated actomyosin-ATPase.
24°, $[K^+ + Na^+] = 0.07$ *mole/lit.*
$[ATP] = 1.17 \times 10^{-3}$ *mole/lit.*
$[MgCl_2] = 1.1 \times 10^{-4}$ *mole/lit.*
(○): veronal-acetate buffer (△): glycine buffer

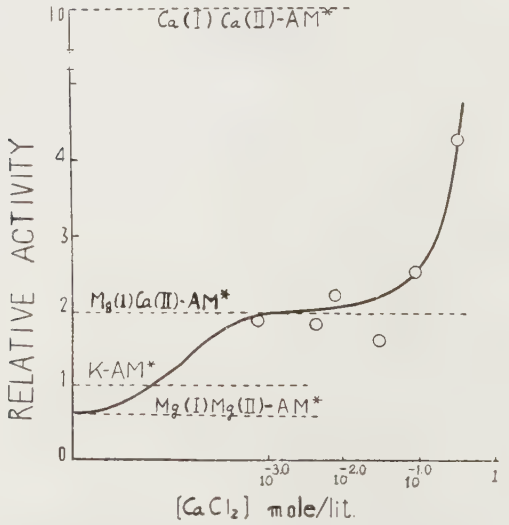


FIG. 5. Activation of actomyosin-ATPase by the addition of Ca^{2+} ion in the presence of Mg^{2+} ion.
pH 6.5 (veronal-acetate), 22°
$[K^+ + Na^+] = 0.09 \sim 0.1$ *mole/lit.*
$[MgCl_2] = 1 \times 10^{-2.0}$ *mole/lit.*

Whatever the mechanism of the above phenomena may be, this activating effect of a small amount of Ca^{2+} on the ATPase action in the presence of Mg^{2+} and K^+ in the physiological concentrations (10^{-2} *M* and 0.1∼0.15 *M* respectively) seems to offer an important key to the understanding of the role of these ions in the muscular contraction. This will be discussed later in this paper.

Some properties of various Mg^{2+}-and Ca^{2+}-actomyosinates in question thus far derived from our experimental results are summarized in Table I.

TABLE I

Complex	p*K*#	Optimum pH	Relative Activity	
			$[K^+ + Na^+] = 0.09 - 0.1 M$	0.15*M*
K-AM*	—		1	1
Mg(I)-AM*	$pK_{Mg(I)} \fallingdotseq 4.5$	7.5	2	0.2
Mg(I)Mg(II)-AM*	$pK_{Mg(II)} \fallingdotseq 2.5$		0.6	0.03
Mg(I)Ca(II)-AM*	$pK_{Ca(II)} > 3.5$		2	0.2
Ca(I)Ca(II)-AM*	$pK_{Ca(I)} \fallingdotseq 2.5$	6.4, 9.7	10	13

$pK_{Mg(I)}$ represents the logarithm of the reciprocal of the dissociation constant for the Mg^{2+} binding at the first ion-binding point and so on.

THE RELATIONSHIP BETWEEN THE CONCENTRATION OF ATP AND THE VELOCITY OF ATP-ASE ACTION

Ouellet *et al.* (*4*) and the present authors (*1*) have already shown that the relationship of the concentration of ATP (less than 10^{-3} *M*) to the ATPase activity (v) of actomyosin in the presence of Ca^{2+} is given by the so-called Michaelis-Menten's formula:

$$v = \frac{V_{max}}{1 + \frac{K_m}{[S]}}$$

Either groups of investigators accord in recognizing that the addition of Mg^{2+} thereto (in the presence of $Ca^{2+} + Mg^{2+}$) leaves *K*m unchanged. This fact means that these ions have no effect on the binding reaction between actomyosin and ATP. The present authors have reinvestigated the relation further in detail and obtained the following results.

Effect of Calcium Ion on Michelis Constant—As can be seen in Fig. 6a, *K*m of actomyosin-ATPase action is nearly doubled by the addition of

Ca^{2+}: Km of K-AM* at pH 6.5 (veronal-acetate), 25°, in the presence of 0.15 M K^+ plus Na^+ is 1.5×10^{-4} *mole/lit.* and that of Ca-AM* is 3.6×10^{-4} and 3.3×10^{-4} *mole/lit.* (in the absence and presence of glycine, respectively).#

It is well known that the Michaelis constant Km is equal to $(k_1{*}'+k_2{*})/k_1{*}$ when the mechanism of ATPase action is as follows:

$$\mathrm{AM{*}+S}\underset{k_1{*}'}{\overset{k_1{*}}{\rightleftharpoons}}\mathrm{AM{*}S}$$

$$\mathrm{AM{*}S}\xrightarrow{k_2{*}}\mathrm{AM{*}+P}$$

Since Ca^{2+} has no effect on the binding reaction between actomyosin and ATP (for the light-scattering experiment, see (*1*) and p. 387 in this report), the increase of Km upon the addition of Ca^{2+} is probably due to the increase of $k_2{*}$. From the values of Vmax, $k_2{*}$ of K-AM* is 0.4 1/*sec.* and $k_2{*}$ of Ca-AM* is 3 1/*sec.* By substituting these $k_2{*}$ values into the above Km, it is calculated that $k_1{*}$ and $k_1{*}'$ are 1.3×10^4 *lit./mole. sec.* and 1.5 1/*sec.* respectively; these values show good agreement with those of the undeformed actomyosin which were obtained from the light-scattering study ($k_1=10\times10^4$ *lit./mole. sec.* and $k_1'=2$ 1/*sec.*, see (*1*) Table VI). Some differences between these values seem to be due to the situation that the light-scattering experiment was conducted in the presence of 0.5 M KCl and, on the other hand, the ATPase activity was measured in the presence of 0.15 M [Na^++K^+]. Ouellet *et al.* (*4*) showed that $k_1'{*}/k_1{*}=1.3\times10^{-5}$ *mole/lit.* in the presence of 0.6 M KCl. This value is in very good agreement with $k_1'/k_1=2\times10^{-5}$ *mole/lit.* in our light-scattering experiment.

As can be seen in Fig. 6a–b, Km of Ca-actomyosinate decreases with the decrease of temperature.##

Effect of higher concentrations of ATP—As shown in Fig. 7, the ATPase activity decreases at ATP concentrations higher than 10^{-3} *mole/lit.*###

Due to the difficulty of estimation of ATPase activity, too much reliance is not allowed as to the accuracy of Km values obtained.

This is incompatible with the result of Ouellet *et al.* (*4*). Their result shows that Km increases with the decreases of temperature. This is probably due to the difference of the Ca^{2+}- and K^+-concentrations applied.

H. H. Weber (*5*) described the similar effect of the ATP concentration on the extracted muscle fiber-ATPase.

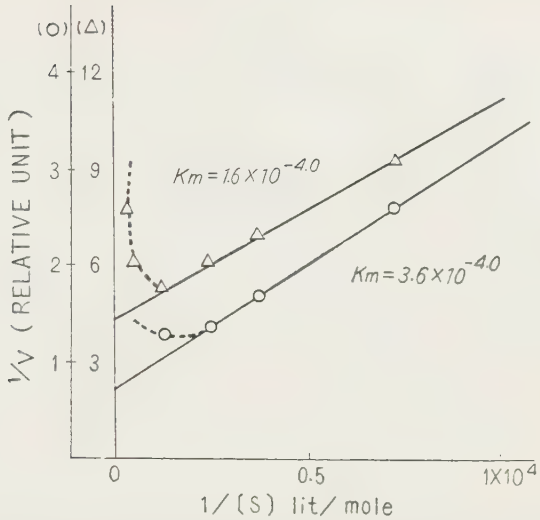


FIG. 6a. Estimation of the Michaelis constant of actomyosin-ATPase (I).
pH 6.5 (veronal-acetate), 25°,
$[K^+ + Na^+] = 0.15$ *mole/lit.* (△)
$[K^+ + Na^+] = 0.15$ *mole/lit.* + $[CaClc_2] = 5 \times 10^{-2}$ *mole/lit.* (○).

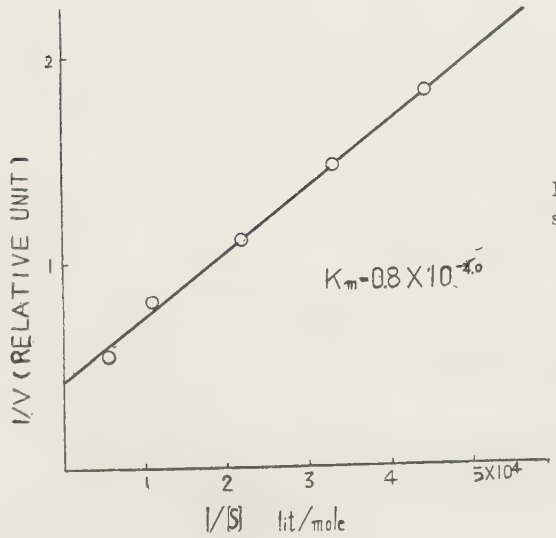


FIG. 6b. Estimation of the Michaelis constant of actomyosin-ATPase (II).
pH 6.3 (veronal-acetate). 6°,
$[K^+ + Na^+] = 1.9 \times 10^{-1}$ *mole/lit.*
$[CaCl_2] = 1.6 \times 10^{-2.0}$ *mole/lit.*
[AM] = 0.35 mg. protein/ml.

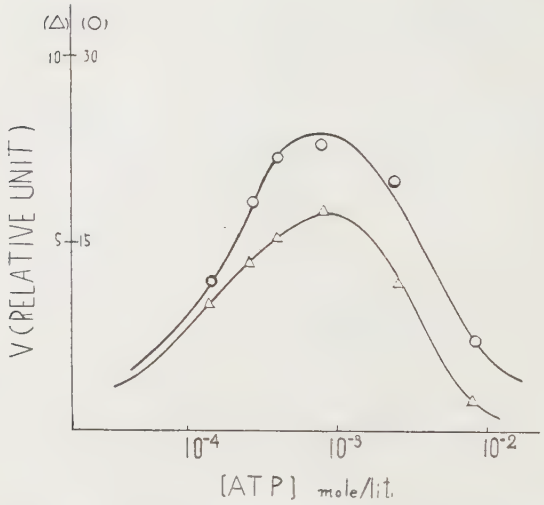


FIG. 7. Relationship between ATPase activity and ATP concentration.
pH 6.5 (veronal-acetate), 25°.,
$[K^+ + Na^+] = 0.15$ *mole/lit.* (△).
$[K^+ + Na^+] = 0.15$ *mole/lit.* $+ [CaCl_2] = 5 \times 10^{-2}$ *mole/lit.* (○).

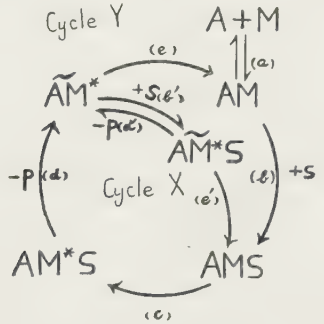
DISCUSSION

In the previous paper (*1*), the interaction between actomyosin and ATP (in the presence of 0.5 *M* KCl) has been represented schematically as follows:

$$\begin{array}{ccccc} & & -P\ \ AM^* & & \\ & (d)\swarrow & & \searrow(e) & \\ AM^*S & & & & AM \rightleftharpoons A+M \\ & (c)\searrow & & \swarrow+S(a) & \\ & & AMS(b) & & \end{array}$$

However, it is well known (*2*) that ATP causes the so-called " superprecipitation " of actomyosin in the presence of about 0.1 *M* KCl, a phenomenon considered to represent a model of muscular contraction and, on the other hand, inorganic pyrophosphate deforms AM to AM* but does not cause the superprecipitation of actomyosin. Considering these facts, it may be natural to presume that upon the splitting of ATP, actomyosin becomes energy-rich and actomyosin in such a state has a certain life; further it seems that the superprecipitation is produced by

the polymerization of energy-rich actomyosin. If this be the case, the interaction of actomyosin to ATP may be shown schematically as follows:



(Affix∼represents the energy-rich state.)

In this schema, the outside cycle Y (b, c, d, e) corresponds to the sequence of a single muscular twitch (see (*1*)) and the inside cycle X (c, d, b′, d′, e′) corresponds to that of the contraction in general. Therefore, if the polymerization of energy-rich actomyosin $\widetilde{AM}^*$ were sufficiently speedy in comparison with the ATPase action (d,d′) and the concentration of the contracted actomyosin [AMcont] is equal to $[\widetilde{AM}^*]$ plus $[\widetilde{AM}^*S]$, the following relationships should hold for the reaction cycle in the presence of the sufficient amount of ATP and Mg^{2+},

$$k_c \gg k_d;\ k_b'\,[S] \gg k_d,\ k_d'.\#$$

Accordingly, in the steady state of reaction,

$$[AMS] \ll [AM^*S],\ [\widetilde{AM}^*] \ll [\widetilde{AM}^*S].$$

That is,

$$[AM_{cont}] \fallingdotseq [\widetilde{AM}^*S],\quad [AM_{rel}]\#\# \fallingdotseq [AM^*S].$$

Hence,

$$\frac{d[AM_{cont}]}{dt} = k_d[AM^*S] - k_e,\ [\widetilde{AM}^*S]$$

$$= k_d[AM_{rel}] - k_e,\ [AM_{cont}] = 0,\#\#\#$$

$$\frac{[AM_{cont}]}{[AM_{rel}]} \fallingdotseq \frac{k_d}{k_e'}$$

---

k_c, k_d, k_b', k_d, are the velocity constants of the reaction steps (c), (d), (b′) and (d′) in the schema proposed above. *Cf.* (*1*), pages 48, 54.

[AM rel] represents the concentration of the relaxed actomyosin.

(### See next page)

As is evident from the equation just derived, any alteration in the condition which would cause any increase in the value of k_d should raise the ratio $[AM_{cont}] / [AM_{rel}]$ and hence lead to a circumstance more favourable for the contraction of actomyosin and *vice versa*.

In the previous paper (*1*), it has been shown that Ca^{2+} and Mg^{2+} exert scarecely influence upon the life of $\widetilde{AM}^*$, *i.e.*, upon the velocity of the reaction $AM^* \rightarrow AM$. Accordingly, it may be deduced that the life of $\widetilde{AM}^*S$, *i.e.*, the velocity constant k_e' of the reaction $\widetilde{AM}^*S \rightarrow AM^*S$ is scarcely affected by these cations. If so, under the ionic conditions in which k_d is large (*i.e.*, the ATPase activity is high), actomyosin will contract, and under the ionic conditions in which ATPase activity is low, actomyosin will relax. In this connection, Korey (*6*) showed that the fibers preserved in glycerol retain their ability to contract when their ATPase activity is above a difinite value and below this level, contraction is absent. Perry (*7*) observed that shortening of myofibrils is most marked at approximately that concentration of $MgCl_2$ which gives optimum ATPase activity.

Recently, Bozler (*8*) has found in the presence of 0.16 *M* KCl and 2 per cent ATP that Mg^{2+} (10^{-2} *M*) was required for the relaxation of the glycerol-treated muscle fiber and that Ca^{2+} in very low concentration (0.5×10^{-3} *M*) produced a rapid contraction. The present authors showed in the former section that the ATPase action of some actomyosin preparations is inhibited by 10^{-2} *M* Mg^{2+} and the addition of 10^{-3} *M* Ca^{2+} thereto results in a reactivation to a level several times (3∼7 times) that of the ATPase activity in the presence of Mg^{2+} alone. Bozler's result may be explained by supposing the same behavior of

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Foot noted (continued)

For convenience's sake, the polymeryzation process $n\widetilde{AM}^*S \rightleftarrows (\widetilde{AM}_{cont})$, the mechanisms of which has not yet been ascertained, is neglected in the present consideration. However, if the muscle contraction is brought about by the polymerization of $n\widetilde{AM}^*S$ and the equilibrium of the polymerization is rapidly established, and if the inactivation process of polymers occurs much slower than that of monomer, so that it may be neglected, the following relations may exist in the steady state:

$$\frac{d\,[AM^*S]}{dt} = k_e'\,[\widetilde{AM}^*S] - k_d\,[AM^*S] = 0, \qquad \frac{[AM^*S]_n}{[AM_{cont}]} = K.$$

Hence

$$\frac{\sqrt[n]{[AM_{cont}]}}{[AM_{rel}]} = \frac{k_d}{k_e'\sqrt[n]{K}}$$

This relation shows that when n is large even a minute change of k_d (or k_e') causes a vast change in [AMcont]/[AM rel].

Mg^{2+} in the reaction of actomyosin-ATPase of their glycerol-treated muscle fibers.

Bozler (*9*) has found also that in the presence of Mg^{2+} ,a high concentration (2 per cent) of ATP was required for the relaxation of the glycerol-treated muscle fiber. This is also in good accordance to our result that the higher concentrations of ATP inhibit the ATPase action.

Contraction of intact muscle seems also not to differ, in this respect, from that in glycerol-treated muscle: Ca^{2+}, at isosmotic concentrations, causes shortening of the intact muscle fiber while Mg^{2+} prevents it (*10*).

It is expected that the simple correspondence of the ATPase activity to the intensity of contraction ($[AM_{cont}]/[AM_{rel}] \fallingdotseq k_d/k_e'$) can be observed only in such a case that the polymerization of energy-rich actomyosin $\widetilde{AM}^*S$ proceeds very rapidly as compared with the ATPase action, while such a correspondence can not be observed when the speed of the polymerization process is slower than that of ATPase action. In this connection, Bowen (*11*, *12*) observed that phosphorylysis does not occur directly concomitantly with the shortening of myosin B threads. Bowen's result is probably an example of the latter case.

SUMMARY

1. Effects of Mg^{2+} and Ca^{2+} on muscle ATPase were investigated in detail. It was noticed that in some actomyosin prparations a small amount of Ca^{2+} (10^{-3} *M*) enhances the ATPase action of actomyosin in the presence of physiological concentrations of K^+ (*ca.* 0.1 *M*) and Mg^{2+} (10^{-2} *M*).

2. At lower concentrations (less than 5×10^{-4} *M*) of ATP, the relation of ATPase activity to the ATP concentration obeys Michelis-Menten's formula. Based on the change of *Km* caused by the addition of Ca^{2+}, the velocity constants k_1^* and $k_1^{*\prime}$, of the binding reaction between the deformed actomyosin (AM*) and ATP (S), *viz.*,

$$AM^* + S \underset{k_1^{*\prime}}{\overset{k_1^*}{\rightleftarrows}} AM^*S$$

were calculated. These calculated values were roughly in accord with those of the binding reaction between ATP and undeformed actomyosin which were estimated in the previous paper (*1*) by measuring the light-scattering change of actomyosin solution.

3. At the concentration of ATP higher than 10^{-3} *M*, the ATPase activity decreases with the increase of the ATP concentration.

4. Comparing these results with the effects of ATP, Ca^{2+} and Mg^{2+} on the contraction and relaxation of the glycerol-treated muscle fiber, it was deduced that the muscular contraction is equivalent to the polymerization of energy-rich actomyosin which is produced by the splitting of ATP.

The authors are indebted to Prof. H. Tamiya, Prof. J. Horiuti and Prof. N. Takasugi for their encouragement and support. Acknowledgement is also made to Messrs K. Yagi, K. Sawafuji and E. Maeda for their valuable assistances in carrying out this research. The present authors have been favoured with Dr. M. F. Morale's and Dr. J. J. Blum's good advices and kind informations. They put it on record as a token of their gratitudes. They are also indebted to Dr. A. Takamiya (Biochemical Laboratory, Tokyo Technical College) for his valuable criticism.

ADDENDUM

After this paper was written, we read the excellent review by Weber and Portzehl (*Advances in Protein Chem.*, **8**, 161 (1952)) entitled "Muscle Contraction and Fibrous Muscle Protein". They have also regarded the breakdown of ATP as the cause of contraction. Their view is chiefly based on the experiments concerning the effects of inorganic polyphosphates and Salyrgan which are non-physiological substances or poisons, while our view is based on the experiments concerning the effects of calcium and magnesium ions in physiological concentrations.

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SUPPLEMETARY REMARKS TO THE "INTERACTIONS BETWEEN ACTOMYOSIN AND ADENOSINETRIPHOSPHATE."

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(Received for pudlication, May 4, 1953)

In the previous papers (*1*, *2*), it was shown that the light-scattering change of actomyosin (AM) solution caused by the addition of ATP (S) is ascribed to the occurrence of the following reactions:

$$AM + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} AMS$$

$$AMS \xrightarrow{k_3} AM^*S.\ddagger$$

Based on this schema, an equation giving the rate of formation of AM*S as a function of substrate concentration [S] and the concentration of actomyosin was derived as follows.:

$$\frac{d[AM^*S]}{dt} = -\frac{d([AM]+[AMS])}{dt} = \frac{k_3([AM]+[AMS])}{1+\frac{k_2+k_3}{k_1[S]}} \qquad \text{Eq. (a)}$$

On that occasion, it was proved experimentally that the above equation applies to the initial course of the change in the light-scattering where $[AM^*S] \ll [AM]+[AMS]$ and [S] is equal to the total concentration of ATP. Generally, [S] as well as [AM]+[AMS] change in the reaction course. Therefore, the authors had not investigated whether or not the total course of the change in the light-scattering obeys the above equation (a).

In the cases where $[AM] \ll [S]$, that is, in such a case as may happen when [S] is scarcely diminished during the course of light-scattering change, the equation (a) can be modified as follows:

$$-\ln\frac{[\Sigma AM]}{([AM]+[AMS])_t} = \frac{k_3}{1+\frac{k_2+k_3}{k_1[S]}} \cdot t \qquad \text{Eq. (b)}$$

‡ AM* represents an deformed actomyosin unit.

where $[\Sigma AM]$ and $([AM]+[AMS])_t$ represent the concentration of total actomyosin and that of undeformed actomyosin at the time t, respectively.

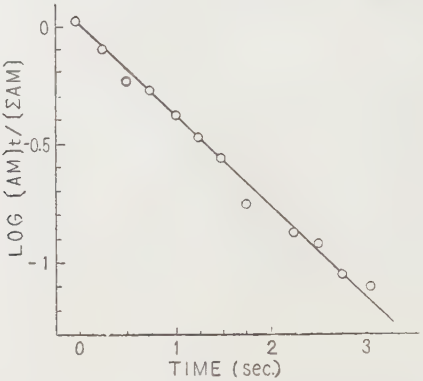

FIG. 1. Relashonship between log $[\Sigma AM]/[AM]+[AMS])_t$ and the time (t). 21° pH 6.4, $[K^+]=0.48$ *mole/lit.* and $[ATP]=1.5\times10^{-4}$ *mole/lit.* $[\Sigma AM]\fallingdotseq10^{-5}$ *mole/lit.*
Tangent of the straight line;
observed $=1/2.7$

$$\text{calculated}=(1/2.31)\times\frac{1}{1+\dfrac{1+2.3}{10^5\times1.5\times10^{-4}}}=1/2.8$$

The present authors ascertained that the Eq. (b) was very well established in their previous experiments which were conducted under such a condition that $[\Sigma AM]\ll[S]$. Fig. 1 shows an example of such a case. The relationship of log $[\Sigma AM]/([AM]+[AMS])_t$ to t is linear and the tangent of the straight line agrees with the calculated values obtained from the above Eq. (b). This result supports the above formula of mechanism proposed by the present authors.

It should be remarked that the reaction unit of actomyosin (AM) is not the unit particle estimated from the hydrodynamical experiments but is the functional one which contains 140,000 gm. of myosin as described in the previous paper (*1*). Recently, Laki and Kominz (*3*) have conducted an amino acid analysis of actin, tropomyosin and myosin. Their results have suggested that myosin is a polymer which is composed of equal mole numbers of actin and tropomyosin. Since

the molecular weights of actin and tropomyosin are 70,000 (*4*) and 65,000 (*5*), respectively, one unit weight of myosin (=tropomyosin+ actin) may be estimated to be about 135,000 gram. This unit weight is in good agreement with that of the functional one of myosin mentioned above. Thus, probably, it may not be too daring to conclude that one component unit of myosin comprises one mole of actin and one mole of tropomyosin and that the one unit contains one active ATP-binding point.

On the other hand, it is known that by the addition of salts, G-actin polymerizes to F-actin and concurrently ATP in G-actin is decomposed to ADP and inorganic phosphate (*6*, *7*). When we compare these changes with the facts known about the interaction of actomyosin with ATP, we find interesting similarities between both phenomena; (i) in both cases, the amount of ATP that is subjected to change is one mole per 70,000 gm. of actin, (*1*, *6*, *8*, *9*) (ii) salts are required for both changes, and (iii) oxidants inhibit the changes reversibly (*10*, *11*, *12*). Sofar, however, no change has been observed when ATP is added to tropomyosin.

On the basis of these facts, it is considered that the active center in actomyosin is in the actin part and is probably identical with the ATP-action point in the G→F transformation. Further, the fact that actin has no activity of ATPase is probably due to the situation that the ATP-binding point is covered on the polymerization of actin.

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STUDIES ON THE FORMIC DEHYDROGENASE OF ESCHERICHIA COLI

III. DETERMINATION OF THE QUANTITY OF THE ENZYME WITHIN THE CELL BY USING HYPOPHOSPHITE AS A SPECIFIC INHIBITOR*

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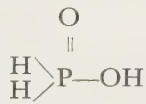
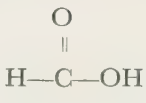
(Received for publication, May 4, 1953)

It has been shown in previous papers**(*1*, *2*, *3*) that the action of formic dehydrogenase in *Escherichia coli* is strongly inhibited by hypophosphorous acid, a substance whose molecular structure has a striking similarity to that of formate:



Hypophosphorous acid



Formic acid

Salient features in the action of this substance were found not only in the strict specificity to the formic dehydrogenase, but also in the marked irreversibility in its reaction with the enzyme. When we denote the concentration of the inhibitor and that of the free enzyme by [G] and [E], respectively, the formation of the inactive enzyme-poison-complex, EG, was found to take place bimolecularly according to the formula:

$$\frac{d\,[EG]}{d\,\tau}=k_g\,[E]\,[G] \qquad (1)$$

where τ is the time of incubation of the enzyme with the poison, and k_g is the velocity constant of the irreversible reaction:

$$E+G\longrightarrow EG \qquad (2)$$

The purpose of the present paper is to show the possibility of de-

* The research was supported in part by the Research Expenditure of the Ministry of Education.

** The present work was preliminarily reported in Japanese (*3*).

termining kinetically the concentration of the formic dehydrogenase in living bacterial cells by taking advantage of both the specificity and irreversibility of the action of said inhibitor.

THEORETICAL

If the concentration of the poison G added is large enough compared with that of the enzyme in the solution, so that the decrease in quantity of the poison due to its combination with the enzyme is negligible, the reaction (2) will proceed in accordance with the law of first order reaction in regard to the enzyme concentration [E]. Under these conditions the constant k_g will be evaluated from the results of experiments in which either the initial concentration of the poison or the length of incubation is varied. That this is actually the case was demonstrated in one of the previous papers. The values of k_g determined in this way for various temperatures and pH-values are summarized in Table I. (*cf.*, (*2*)).

Table I

pH	Temp.	k_g	pH	Temp.	k_g	pH	Temp.	k_g
3.4	43°	4900	6.0	0°	21.0	7.0	10°	17.7
„	30°	1380	7.0	50°	54.8	„	0°	9.1
„	10°	203	„	45°	280.	8.5	43°	179.
5.0	30°	385	„	30°	128.	„	30°	104.
6.0	30°	262	„	20°	40.8	„	10°	13.0
„	15°	69.0	„	15°	30.5	„	0°	5.7

When, on the other hand, the concentration of the poison added to the enzyme solution is very low and comparable to that of the enzyme used, the concentration [G] of the poison existing in the mixture cannot be regarded as being constant during the incubation time. In this case, Eq. (1) must be written in the following form:

$$\frac{d\,[EG]}{d\,\tau}=k_g\,(\varepsilon-[EG])\,(g-[EG])$$

or

$$\frac{d\,[EG]}{(\varepsilon-[EG])\ (g-[EG])}=k_g\ d\tau \qquad (3)$$

where ε and g represent the initial concentrations of the enzyme and the poison, respectively; namely:

$$\left.\begin{aligned}\varepsilon&=[E]+[EG]\\ g&=[G]+[EG]\end{aligned}\right\} \qquad (4)$$

Now, let us consider a special case in which the initial concentration of the poison is equal to that of the enzyme, *i.e.*

$$g=\varepsilon$$

Then, Eq. (3) is simplified to

$$\frac{d\,[EG]}{(\varepsilon-[EG])^2}=k_g\ d\tau$$

Taking into consideration the fact that the concentration of the complex EG is equal to zero at the beginning of the incubation ($\tau=0$), we have by integration of the above equation:

$$\frac{[EG]}{\varepsilon\ (\varepsilon-[EG])}=k_g\tau \qquad (5)$$

In a previous paper (*3*), it was shown that the quantity of the free enzyme existing at each time during the incubation with the inhibitor can be estimated by adding to the mixture a sufficiently high concentration of formic acid and by determining the velocity of its dehydrogenation. This was explained as being due to the situation that formic acid, in high concentrations, will occupy the whole existing free enzymes, at the moment of its addition, thus preventing the latter from being further acted upon by the molecules of the poison. If we denote by V_g the velocity of dehydrogenation of formate under such conditions (*i.e.*, in the presence of certain amount of poison and of a sufficiently high concentration of formate), then we may write

$$V_g=k_1[E]=k_1\ (\varepsilon-[EG]) \qquad (6)$$

In the absence of the poison, $[EG]=0$, so that we have

$$V=k_1\varepsilon \qquad (7)$$

where V is the velocity of dehydrogenation of formate in the absence of the poison and in the presence of sufficiently high concentration of formate.

If we define the degree of inhibition (H) by

$$H=1-\frac{V_g}{V}$$

we have from Eqs. (6) and (7),

$$H=\frac{[EG]}{\varepsilon} \tag{8}$$

Taking into consideration Eqs. (5), (6), (7) and (8), we have

$$\frac{H}{1-H}=k_g\, g\, \tau$$

or

$$\frac{H}{(1-H)k_g\, g\tau}=1 \tag{9}$$

It should be noticed that this relation will hold true only under the condition where $g=\varepsilon$. By comparing this equation with Eq. (3), it will be seen that the value $\frac{H}{(1-H)k_g g\tau}$ will be larger or smaller than unity according as g is larger or smaller than ε. k_g is the value which we have determined in our previous report, while H is a value which can be measured experimentally for each desired length of incubation. By determining these values for different concentrations of the poison, we may find the value of g at which $\frac{H}{(1-H)k_g\, g\, \tau}$ equals unity. This value of g may be regarded as representing the amount of the enzyme existing in the experimental solution used.

EXPERIMENTAL METHODS

Escherichia coli was grown on an ordinary peptone-broth-agar at 30°. After 18 hours of culture, the cells were harvested, washed three times with distilled water and suspended in a solution of the following composition,

M/60 Phosphate buffer (pH 7,0)

$0.125\sim1.0\times10^{-6}$ *M* Potassium hypophosphite.

0.659 mg./ml. Bacteria (dry weight)

(Control tests were run without hypophosphite).

The suspension was incubated in a thermostat at 20° and, at appropriate intervals, 4.5 ml. each of the mixture were taken out and mixed immediately with 3.0 ml. of the formate solution (consisting of *M*/16 of phosphate mixture, *M*/4 of formic acid, adjusted to pH 7.0 with

sodium hydroxide). With 2.5 ml. of the resulting mixture, the remaining enzymatic activity was measured in terms of the rate of O_2 uptake by using the Warburg manometer at 30°. The rate of oxygen uptake was found to be fairly constant within more than 40 minutes of observation, so that the reaction velocity V or V_g (Eqs. 6 and 7) could be determined with sufficient accuracy.

TABLE II

g	τ (*seconds*)	V (*mm.³/hour*)	V_g (*mm.³/hour*)	H	$\frac{H}{(1-H)\,k_{gg}\,\tau}$
1.0×10^{-6}	7440	160.0	111.0	0.307	1.46
	"	"	115.0	0.281	1.29
	15300	136.6	78.0	0.429	1.20
	"	"	76.0	0.443	1.28
0.5×10^{-6}	11460	146.3	112.2	0.232	1.29
0.25×10^{-6}	11460	146.3	130.0	0.112	1.08
	26160	119.2	94.4	0.208	0.985
	"	"	96.0	0.195	0.910
	36600	103.6	77.4	0.253	0.908
	"	"	78.0	0.248	0.884
0.20×10^{-6}	22380	124.7	112.8	0.096	0.581
	"	"	106.2	0.147	0.943
0.16×10^{-6}	19080	129.4	120.0	0.072	0.622
	26160	119.2	103.6	0.131	0.883
	"	"	107.0	0.103	0.673
0.125×10^{-6}	15300	136.6	129.0	0.055	0.744
	22380	124.7	117.6	0.057	0.529
	"	"	119.0	0.046	0.421
	29640	114.0	101.4	0.110	0.818
	"	"	103.6	0.091	0.662
	36600	103.6	95.7	0.076	0.441
	"	"	95.0	0.083	0.485

RESULTS

The results of experiments are summarized in Table II and Figs. 1 and 2.

The dotted lines in Fig. 2 represent the theoretical values of H which would be obtained if the concentration of the poison added was exactly equal to that of the enzyme. As is apparent from the figure,

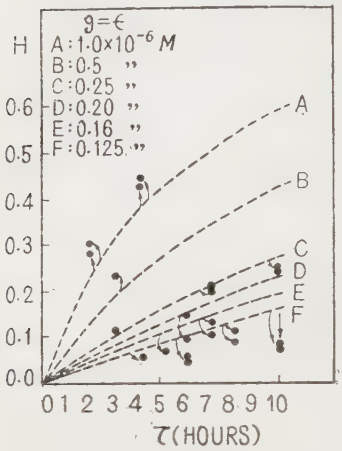


Fig. 1. Progressive inhibition of formic dehydrogenase of *Escherichia coli* by hypophosphite in concentrations comparable to that of the enzyme. H: grade of inhibition. pH 7.0, 20°. Explanation in text.

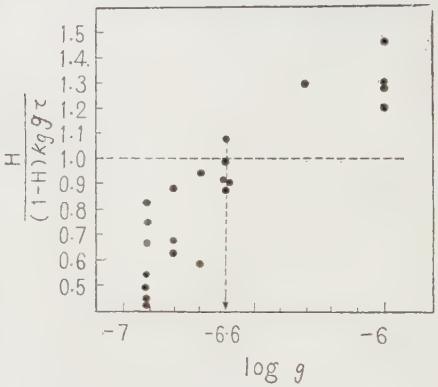


Fig. 2. Inhibition of formic dehydrogenase of *Escherichia coli* by hypophosphite. Explanation in text (see Eq. (9)).

the value of H fell above or below the dotted line according as g was greater or smaller than a certain value, the approximate coincidence between the theoretical and experimental values having been observed only in the case c (Fig. 1) where g was equal to $0.25\ 10^{-6}$ mole/lit. In Fig. 2 the value $H/(1-H)k_g\ g\tau$ is plotted against log g. The fact that the former value approximately equals unity at log $g=-6.6$ indicates that the bacterial suspension used in this experiment contained the enzyme in an concentration of

$$0.25\times10^{-6}\ \text{mole/lit.}$$

Since the suspension used contained 0.695 mg. (dry weight) of bacteria, it follows that one gram of the bacteria contained

$$\frac{0.25\times10^{-6}}{0.659}=3.8\times10^{-7}\ \text{g. mole of the enzyme.}$$

From the value of ε given above and that of the velocity (V) of O_2-uptake in the absence of the poison (see Table II), the absolute value of the velocity constant k_1 (Eq. 8) can now be computed as follows:

Velocity of O_2-uptake:

$V = 160$ mm^3/hr. per manometer cup.*

$= \frac{7.14 \times 10^{-6}}{2.5 \times 10^{-3}}$ mole O_2/lit. hr.

$= 2.85 \times 10^{-3}$ mole O_2/lit. hr.

Enzyme concentration:

$$(\varepsilon) = 0.25 \times 10^{-6} \times \frac{4.5}{3.0 + 4.5}$$

$= 1.5 \times 10^{-7}$ mole/lit.

Velocity constant:

$k_1 = V/\varepsilon$

$= 1.9 \times 10^4$ molecules of O_2 per hr. per molecule of enzyme.

$= 5.3$ molecules of O_2 per sec. per molecule of enzyme.

(or 10.6 molecules of formic acid per sec. per molecule of enzyme)

DISCUSSION AND CONCLUSION

It should be noticed that the whole theoretical considerations developed above are based on the following assumptions:

a) The inhibition of the enzyme was caused by a stoichiometric and irreversible reaction between the enzyme and the poison, as is indicated by Eq. (1).

b) During the period of observation, the reaction between the enzyme and the poison represented the only cause of the depression of the enzyme activity, and no other contingent process occurred which would bring about the poisoning of the enzyme.

c) In the bacterial cells, the enzyme in question represented the only site of attack by the poison, and no other cellular component had combined with it.

Evidences for the fulfillment of the conditions (a) and (b) were already afforded by the experiments reported in our previous works. As to the point (c), there is no direct evidence available at present.

* See the value for V in the first line in Table II.

The fact, however, that the time course of the increase of poisoning obeys satisfactorily the rule predicted from the scheme assumed seems to warrant the validity of our assumption.

The figure 3.8×10^{-7} g. mole (per one g. bacteria) we have attained in our lines of reasoning may, therefore, be regarded as representing a value, at least approximate, if not exactly accurate, for the concentration of the enzyme in the cells of *Escherichia coli*.

On the basis of the value of ε thus obtained, the absolute value for the velocity constant k_1 was computed to be 10.6 (molecules of formic acid per second per molecule of enzyme) under the conditions of the experiment (pH 7.0, 30°).

The author wishes to acknowledge his indebtedness to Prof. H. Tamiya of the University of Tokyo for his kind guidance and encouragement in carrying out this research.

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STUDIES ON THE FORMIC DEHYDROGENASE OF ESCHERICHIA COLI

IV. THE KINETICS OF THE COMPETITIVE INHIBITION BY HYPOPHOSPHOROUS ACID*

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(Received for publication, May 4, 1953)

Previously published experiments (*1*, *2*, *3*) have shown that the reaction of formic dehydrogenase of *Escherichia coli* in the presence of formic acid and hypophosphorous acid may be represented by the following equations:

$$E + G \xrightarrow{k_g} EG \qquad (1)$$

$$E + S \underset{k'}{\overset{k}{\rightleftharpoons}} ES \xrightarrow{k_1} E + P \qquad (2)$$

where E denotes the enzyme, G the poison, S the substrate, ES and EG the complexes of the enzyme with the substrate and the poison, respectively, and P the products of oxidation of formate in the presence of hydrogen acceptor such as methylene blue or oxygen. k's are the velocity constants of each step indicated.

Characteristics of this inhibitory phenomenon lie in the facts (i) that the poison and the substrate compete with each other for the enzyme, (ii) that the complex EG is not capable of combining with the substrate, and the complex ES is not affected by the poison, and (iii) that reaction (1) proceeds relatively slowly, while reaction (2) takes place very rapidly. Owing to the concurrence between G and S for E, the reaction between E and G is more or less hindered by the presence of the substrate. It has been shown in previous works, that the progress of poisoning was practically completely halted at the moment of addition of formate in sufficient concentrations, provided that the concentration of the poison was not too high. In the presence of

* The research was supported in part by the Research Expenditure of the Ministry of Education.

higher concentrations of the poison, and relatively lower concentration of the substrate, the possibility remains for the poison to react with the free enzyme molecule E existing in the reaction mixture. According to Eq. (2), the concentration of E in the stationary state may be expressed by

$$[E]=\frac{[E]+[ES]}{1+K/[S]} \tag{3}$$

$$K=\frac{k'+k_1}{k} \tag{4}$$

where K is the Michaelis constant of the enzyme. The purpose of the present investigation is to study the kinetics of the progressive poisoning to be observed under such conditions.

THEORETICAL

According to reaction (1) the reaction of the poison with the enzyme takes place with the velocity:

$$\frac{d\ [EG]}{d\ t}=k_g[E]\ [G] \tag{5}$$

In the presence of the substrate, the stationary state of the reaction between the enzyme and the substrate is attained almost instantaneously according to Eq. (3) and this relationship may be considered to hold, at any moment, irrespective of whether the poison is present or not. If the total concentration of the enzyme is ε, the rate of formation of EG in the presence of S is

$$\begin{aligned}\frac{d\ [EG]}{d\ t}&=k_g[G]\ [E]\\ &=\frac{d\ (\varepsilon-([ES]+[E]))}{dt}\end{aligned} \tag{6}$$

From (3) and (6) we have

$$\frac{d\ \{\varepsilon-[ES]\ (1+K/[S])\}}{[ES]}=\ k_g\frac{[G]}{[S]}K\ dt$$

If $[EG]=0$ at $t=0$, the integration of the above equation gives:

$$\ln\frac{\varepsilon}{[ES]}\cdot\frac{1}{1+K/[S]}=\ k_g[G]\frac{1}{1+[S]/K}t \tag{7}$$

Under the condition where [S] is sufficiently large, so that $K/[S]$ is negligibly small compared with 1, we have from Eq. (7),

$$\ln\ (V_g/V) = -k_g K \frac{[G]}{[S]} t \qquad (8)$$

where V_g and V represent the velocities of the enzyme reaction in the presence and absence of the poison, respectively.

If we denote the degree of inhibition by H and define it by

$$H = \frac{V - V_g}{V},$$

then we obtain the equation:

$$\ln\ (1-H) = -k_g K \frac{[G]}{[S]} t \qquad (9)$$

In the following it will be shown that this equation fits in satisfactorily with the data obtained under the experimental condition specified in the above discussion.

METHODS

A 16-hour culture of *Escherichia coli* on peptone-broth-agar was washed twice with distilled water and suspended in a reaction medium of the following composition:

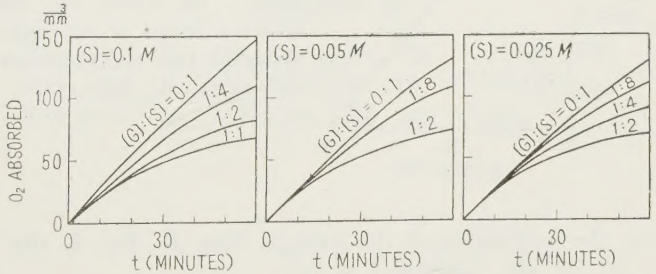
1.0 ml. bacterial suspension (containing 1.35 mg. dry weight of bacteria)

0.5 ml. phosphate buffer (pH 7.0, *M*/4)

0.5 ml. formate solution, neutralized with sodium hydroxide.)

0.5 ml. potassium hypophosphite (or distillated water, in control experiments).

Final concentrations of the substrate and the poison were varied



FIGS. 1, 2, and 3. Competitive inhibition of formic dehydrogenase of *Escherichia coli* by hypophosphite in concentrations [G] comparable to that of the substrate [S]. pH 7.0, 30°.

as indicated under the individual experiments.

The oxidation of formate was followed manometrically with the Warburg manometer at 30°.

RESULTS

The representative results of experiments are shown in Figs. 1 to 3. From these figures it may be seen, that while the velocity of the reaction in the absence of the poison remains fairly constant during the course of the reaction of an hour or more, the reaction rate in the presence of the poison shows a progressive decline with the lapse of time. It may further be noticed by comparison of the three figures that the time course of the reaction is determined by the ratio of the concentration of the poison to that of the substrate ([G]/[S]) and not by the absolute concentrations of each of these substances. This is just what has been predicted from the theory. (Eq. (8)).

Satisfactory agreement between the theory and experiment was also obtained for the phenomenon of gradual increase of poisoning with the lapse of time. In accordance with Eq. (9), the logarithm of the grade of survival $(1-H)$ was found to be proportional to the time t, and also to the ratio [G]/[S], as it is demonstrated in Fig. 4. which reproduces the result of the experiment given in Fig. 1.

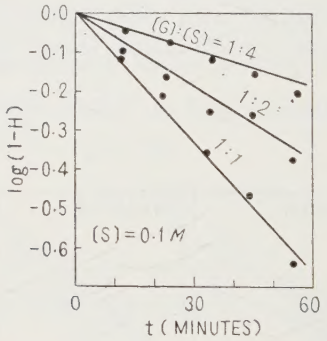


FIG. 4. Competitive inhibition of formic dehydrogenase of *Escherichia coli* by hypophosphite. The values are taken from the results of experiments given in Fig. 1. (Explanation in text (see Eq. 9)).

From the inclination of the straight lines in Fig. 4, the value of k_gK (see Eq. 9) was computed to be:

$$k_gK = 0.00046 \text{ (sec.}^{-1}\text{)}$$

In the previous paper, the Michaelis constant K of the enzyme

was estimated to be

$K = 10^{-3.3}$ mole/lit. = 0.0005 mole/lit. (pH 7.0, 30°).

From these two values we can calculate the magnitude of k_g as follows:

$k_g = 0.92$ (mole^{-1}, lit., sec.$^{-1}$)

In our previous paper (*3*), the value of k_g was determined by an experiment using lower concentrations (of the order of 10^{-5} mole/lit.) of the poison. The figures found were remarkably higher (*cf.*, (*3*) Table I) than that given above. The question as to why this discrepancy was caused is left open to further investigations.

SUMMARY

The kinetics of the inhibitory action of hypophosphorous acid on the formic dehydrogenase of *Escherichia coli* was studied. The progressive inhibition of the enzyme in the presence of higher concentrations of the poison together with the substrate was followed and the results were shown to be in accordance with the reaction scheme previously proposed by the author.

Based on the experimental data, the velocity constant (k_g) of the reaction between the poison and the enzyme was computed, and it was found to be noticeably lower than the value obtained in the previous work (*2*) for lower concentrations of the poison.

The author's thanks are due to Prof. H. Tamiya of the University of Tokyo for his encouragement and support in carrying out this research.

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